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(54) Title: IMAGING, DIAGNOSIS AND TREATMENT OF DISEASE



WO 02/36771 A2

	HCT116 SW480 PEK 4 hypoxia PEK 4 normoxia HeLa MDA 468 MDA 453 NES hypoxia NES normoxia HUVEC proliferating HUVEC confluent HMVEC
ECSM1 - Hs. 13957	
magic roundabout	
Hs.233955	
fatty acid binding protein 4	
von Willebrand factor	
serum deprivation response protein	
collagen, type IV, alpha 1	
EGF-containing fibulin-like extracellular matrix protein 1	
connective tissue growth factor	

(57) Abstract: The present invention relates to endothelial cell-specific genes and encoded polypeptides and materials and uses thereof in the imaging, diagnosis and treatment of conditions involving the vascular endothelium.



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IMAGING, DIAGNOSIS AND TREATMENT OF DISEASE

The present invention relates to genes whose expression is selective for the endothelium and use of these genes or gene products, or molecules which bind thereto, in imaging, diagnosis and treatment of conditions involving the vascular endothelium.

The endothelium plays a central role in many physiological and pathological processes and it is known to be an exceptionally active transcriptional site. Approximately 1,000 distinct genes are expressed in an endothelial cell. In contrast red blood cells were found to express 8, platelets 22 and smooth muscle 127 separate genes (Adams *et al*, 1995). Known endothelial specific genes attract much attention from both basic research and the clinical community. For example, the endothelial specific tyrosine kinases Tie, TIE2/TEK, KDR, and flt1 are crucial players in the regulation of vascular integrity, endothelium-mediated inflammatory processes and angiogenesis (Sato *et al*, 1993, Sato *et al*, 1995, Fong *et al*, 1995, Shalaby *et al*, 1995, Alello *et al*, 1995). Angiogenesis is now widely recognised as a rate-limiting process for the growth of solid tumours. It is also implicated in the formation of atherosclerotic plaques and restenosis. Finally endothelium plays a central role in the complex and dynamic system regulating coagulation and hemostasis.

Of the many distinct genes expressed in an endothelial cell, not all are entirely endothelial cell selective and so the genes and their products, and molecules which bind thereto are not generally useful in the imaging, diagnosis and treatment of disease. Thus, there remains a need for endothelial cell specific or selective molecules.

We report here identification of two highly endothelial selective genes which we have called: endothelial cell-specific molecule 1 (ECSM1) and magic roundabout (endothelial cell-specific molecule 4; ECSM4). The terms ECSM1
5 and ECSM4 are also used to indicate, as the context will make clear, the cDNA and polypeptides encoded by the genes. These genes, and especially ECSM4, are surprisingly specific in their cell expression profile. ECSM4, for example, shows similar endothelial-cell selectivity to the marker currently accepted in the art as the best endothelial cell marker (von Willibrand Factor). Clearly,
10 such a high level of endothelial cell specificity is both unprecedented and unexpected.

ECSM1 (UniGene entry Hs.13957) has no protein or nucleotide homologues. It is most likely to code for a small protein of 103 aa (the longest and most up-
15 stream open reading frame which was identified in the contig sequence). ECSM1 contains two sequence tagged sites which are unique and definite within the genome (STS sites; dbSTS G26129 and G28043) and localise to chromosome 19. A polynucleotide comprising the complement of part of the ECSM1 gene is described in WO 99/06423 (Human Genome Sciences)
20 (termed "gene 22"; page 31-32) as being expressed primarily in umbilical cord endothelial cells and to a lesser extent in human adipose tissue. However, WO 99/06423 discloses an open reading frame (ORF) in the polynucleotide which encodes a polypeptide of only 45 amino acids. According to our analyses, this does not represent the correct polypeptide of 103 amino acids, as the actual
25 start codon in ECSM1 is further 5' than the one identified in WO 99/06423.

The human magic roundabout (ECSM4) cDNA clone with a long ORF of more than 417 aa (GenBank Accession No AK000805) and described in WO 99/46281 as a 3716 nucleotide sequence was identified by BLAST searches for the Hs.111518 contig. This sequence is rich in prolines and has several regions of low amino acid complexity. BLAST PRODOM search (protein families database at HGMP, UK) identified a 120 bp region of homology to the cytoplasmic domain conserved family of transmembrane receptors involved in repulsive axon guidance (ROBO1 DUTT1 protein family, $E=4e-07$). Homology was extended to 468 aa ($E=1.3e-09$) when a more rigorous analysis was performed using ssearch (Smith and Waterman 1981) but the region of similarity was still contained to the cytoplasmic domain. The ROBO1 DUTT1 family comprises the human roundabout homologue 1 (ROBO1), the mouse gene DUTT1 and the rat ROBO1 (Kidd *et al*, 1998, Brose *et al*, 1999). Because of this region of homology we called the gene represented by Hs. 111518 "magic roundabout" (ECSM4). Additionally, BLAST SBASE (protein domain database at HGMP) suggested a region of similarity to the domain of the intracellular neural cell adhesion molecule long domain form precursor ($E=2e-11$). It should be noted that the true protein product for magic roundabout is likely to be larger than the 417 aa coded in the AK000805 clone since the ORF has no apparent up-stream limit, and size comparison to human roundabout 1 (1651 aa) suggests a much bigger protein. This is confirmed in Figure 3 which shows the translation product of human ECSM4 to be around 118kDa. However, ECSM4 is smaller than other members of the roundabout family, sharing only two of the five Ig domains and two of the three fibronectin domains in the extracellular region. The intracellular putative proline rich region that is homologous to those in roundabout are thought to couple to c-abl. Figure 12 shows the full length

amino acid sequence of human ECSM4 (1105aa), and the sequence of the mouse homologue is shown in Figure 13. Nucleotide coding sequences which display around 99% identity to the ECSM4 nucleotide sequence given in Figure 12 are disclosed in WO 99/11293 and WO 99/53051.

5

Additional sequences which display homology to the ECSM4 polypeptide or polynucleotide sequence are disclosed in EP 1 074 617, WO 00/53756, WO 99/46281, WO 01/23523 and WO 99/11293. However, none of these publications disclose that the sequences are selectively expressed in the
10 vascular endothelium, nor suggest that they may be so expressed.

Recently intriguing associations between neuronal differentiation genes and endothelial cells have been discovered. For example, a neuronal receptor for vascular endothelial growth factor (VEGF) neuropilin 1 (Soker *et al*, 1998) was
15 identified. VEGF was traditionally regarded as an exclusively endothelial growth factor. Processes similar to neuronal axon guidance are now being implicated in guiding migration of endothelial cells during angiogenic capillary sprouting. Thus ephrinB ligands and EphB receptors are involved in demarcation of arterial and venous domains (Adams *et al*, 1999). It is possible
20 that magic roundabout (ECSM4) may be an endothelial specific homologue of the human roundabout 1 involved in endothelial cell repulsive guidance, presumably with a different ligand since similarity is contained within the cytoplasmic i.e. effector region and guidance receptors are known to have highly modular architecture (Bashaw and Goodman 1999).

25

However, to date there has been no mention of the existence of an endothelial counterpart, nor the expression pattern of the magic roundabout (ECSM4) gene

being restricted to endothelial cells especially angiogenic endothelial cells, nor of any function of the encoded polypeptide.

It should be noted that a surprising result of our RT-PCR analysis, described in Example 1, was that genes identified here appear to show endothelial specificity (Fig. 1) comparable with the classic endothelial marker von Willebrand factor (vWF). Expression of known endothelial specific genes is not usually 100% restricted to the endothelial cell. Data presented herein shows the quite unanticipated finding that ECSM4 is not expressed at detectable levels (at least using the methods described in the examples) in cell types other than endothelial cells, given the less than 100% selectivity of known endothelial cell markers. Ribonuclease protection analysis has confirmed and extended this observation (Figure 14a). ECSM4 expression was seen to be restricted to endothelium (three different isolates) and absent from fibroblast, carcinoma and neuronal cells. KDR and FLT1 are both expressed in the male and female reproductive tract: on spermatogenic cells (Obermair *et al*, 1999), trophoblasts, and in decidua (Clark *et al*, 1996). KDR has been shown to define haematopoietic stem cells (Ziegler *et al*, 1999). FLT1 is also present on monocytes. In addition to endothelial cells vWF is strongly expressed in megakaryocytes (Sporn *et al*, 1985, Nichols *et al*, 1985), and in consequence present on platelets. Similarly, multimerin is present both in endothelial cells (Hayward *et al*, 1993) and platelets (Hayward *et al*, 1998).

Generally speaking, endothelial and haematopoietic cells descend from same embryonic precursors: haemangioblasts and many cellular markers are shared between these two cell lineages (for review see Suda *et al*, 2000). Hence, the

finding that the genes ECSM1 and ECSM4 are not expressed in cells other than those of the vascular endothelium is highly surprising.

Determination of genes whose expression is selective for the vascular endothelium allows selective targeting to these cells and thereby the specific
5 delivery of molecules for imaging, diagnosis, prognosis, treatment, prevention and evaluation of therapies for conditions associated with normal or aberrant vascular growth.

10 A first aspect of the invention provides a compound comprising (i) a moiety which selectively binds the polypeptide ECSM4 and (ii) a further moiety.

By "the polypeptide ECSM4" we include a polypeptide whose sequence comprises or consists of the amino acid sequence given in Figure 4 or 5 or 7 or
15 12 or 13 or whose sequence is encoded by the nucleotide sequence given in Figure 4 between nucleotides 1 and 1395 or between nucleotides 2 and 948 of Figure 5 or Figure 7 or between nucleotides 71 and 3442 of Figure 12 or between nucleotides 6 and 3050 of Figure 13 and natural variants thereof. Preferably, the ECSM4 polypeptide is one whose amino acid sequence
20 comprises the sequence given in Figure 4 or Figure 12.

By "the polypeptide ECSM4" we include a polypeptide represented by SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or
25 SEQ ID No 86 of WO 99/11293, or the polypeptide represented by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00/53756 or

WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of WO 99/11293.

By "the polypeptide ECSM4" we also include any naturally occurring
5 polypeptide which comprises a consecutive 50 amino acid residue portion or natural variants thereof of the polypeptide sequence given in Figure 4 or 5 or 7 or 12 or 13. Preferably, the polypeptide is a human polypeptide.

Embodiments and features of this aspect of the invention are as described in
10 more detail below.

A second aspect of the invention provides a compound comprising (i) a moiety which selectively binds the polypeptide ECSM1 and (ii) a further moiety.

15 Preferably, in the first and second aspects of the invention, the binding moiety and further moiety are covalently attached.

By "the polypeptide ECSM1" we include a polypeptide whose amino acid sequence comprises or consists of the sequence given in Figure 2 and natural
20 variants thereof.

By "the polypeptide ECSM1" we also include any naturally occurring polypeptides which comprises a consecutive 50 amino acid residue portion or natural variants thereof of the polypeptide sequence given in Figure 2.
25 Preferably, the polypeptide is a human polypeptide.

Preferably, the polypeptide ECSM1 amino acid sequence comprises the sequence given in Figure 2 but does not comprise the amino acid sequence encoded by ATCC deposit No 209145 made on July 17 1997 for the purposes of WO 99/06423.

5

By "natural variants" we include, for example, allelic variants. Typically, these will vary from the given sequence by only one or two or three, and typically no more than 10 or 20 amino acid residues. Typically, the variants have conservative substitutions.

10

In a preferred embodiment of the first or second aspects of the invention, the moiety capable of selectively binding to the specified polypeptide is an antibody.

15 Preferably, an antibody which selectively binds ECSM1 or a natural variant thereof is not one which binds a polypeptide encoded by SEQ ID No 32 of WO 99/06423 or encoded by the nucleic acid of ATCC deposit No 209145 made on July 17 1997 for the purposes of WO 99/06423.

20 Preferably, an antibody which selectively binds ECSM1 is one which binds a polypeptide whose amino acid sequence comprises the sequence given in Figure 2 or a natural variant thereof but which polypeptide does not comprise the amino acid sequence encoded by ATCC deposit No 209145 made on July 17 1997.

25

Preferably, an antibody which selectively binds ECSM4 is one which selectively binds a polypeptide with the sequence GGDSLLGGRGSL,

LLQPPARGHAHDGQALSTD, EPQDYTEPVE, TAPGGQGAPWAE or
ERATQEPSEHGP or a sequence which is located in the extracellular portion
of ECSM4. As described in more detail below, these sequences represent
amino acid sequences which are only found in the human ECSM4 and are not
5 found in the mouse ECSM4 polypeptide sequence.

Preferably, the moiety which selectively binds ECSM4, such as an antibody,
is one which binds a polypeptide whose amino acid sequence comprises the
sequence given in any one of Figures 4, 5, 7, 12 or 13 or a natural variant
10 thereof but does not bind the polypeptide represented by any one of SEQ ID
No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or
WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of
WO 01/23523, or SEQ ID No 86 of WO 99/11293, or encoded by any one
of the nucleotide sequences represented by SEQ ID No 18084 or 5096 of
15 EP 1 074 617, SEQ ID No 210 of WO 00 53756 or WO 99/46281, or SEQ
ID Nos 22, 23, 96 or 98 of WO 01/23523 and SEQ ID No 31 of
WO 99/11293.

By "antibody" we include not only whole immunoglobulin molecules but also
20 fragments thereof such as Fab, F(ab')₂, Fv and other fragments thereof that
retain the antigen-binding site. Similarly the term "antibody" includes
genetically engineered derivatives of antibodies such as single chain Fv
molecules (scFv) and domain antibodies (dAbs). The term also includes
antibody-like molecules which may be produced using phage-display
25 techniques or other random selection techniques for molecules which bind to
ECSM1 or ECSM4.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains
5 of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of
10 the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible
15 oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**,
20 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

25 The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration to the target site. Effector

functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

5

Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we mean that the said antibodies and F(ab')₂ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining site.

10

Although the antibody may be a polyclonal antibody, it is preferred if it is a monoclonal antibody. In some circumstance, particularly if the antibody is going to be administered repeatedly to a human patient, it is preferred if the monoclonal antibody is a human monoclonal antibody or a humanised monoclonal antibody.

15

Suitable monoclonal antibodies which are reactive as said may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies; A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and Application*", SGR Hurrell (CRC Press, 1982). Polyclonal antibodies may be produced which are polypepcific or monospecific. It is preferred that they are monospecific.

20

Chimaeric antibodies are discussed by Neuberger *et al* (1998, 8th International Biotechnology Symposium Part 2, 792-799).

25

Suitably prepared non-human antibodies can be "humanised" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies.

- 5 The antibodies may be human antibodies in the sense that they have the amino acid sequence of human anti-ECSM1 or -ECSM4 antibodies but they may be prepared using methods known in the art that do not require immunisation of humans. For example, transgenic mice are available which contain, in essence, human immunoglobulin genes (see Vaughan *et al* (1998) *Nature Biotechnol.*
10 16, 535-539.

In an alternative embodiment, the moiety capable of selectively binding to a polypeptide is a peptide. The ECSM4/magic roundabout polypeptide shows homology with the *Drosophila*, mouse and human roundabout proteins, which
15 are cell surface receptors for secreted Slit proteins (Li *et al* (1996) *Cell* 96:807-818). Any cognate ligand for ECSM4/magic roundabout which is capable of selectively binding the region of the polypeptide which is located extracellularly may be useful. The extracellular region of ECSM4 is likely to be located within residues 1-467 of the ECSM4 polypeptide sequence given in
20 Figure 12. It is believed that certain peptides may be cognate ligands for ECSM4. Such a peptide will be a suitable moiety for selectively binding ECSM4/magic roundabout. Peptides binding ECSM4 can be identified by means of a screen. A suitable method or screen for identifying peptides or other molecules which selectively bind ECSM4 may comprise contacting the
25 ECSM4 polypeptide with a test peptide or other molecule under conditions where binding can occur, and then determining if the test molecule or peptide has bound ECSM4. Methods of detecting binding between two moieties are

well known in the art of biochemistry. Preferably, the known technique of phage display is used to identify peptides or other ligand molecules which bind to ECSM4. An alternative method includes the yeast two hybrid system.

- 5 Peptides or other agents which selectively bind ECSM4 include those which modulate or block the function of ECSM4.

Suitable peptides may be synthesised as described in more detail below.

- 10 The further moiety may be any further moiety which confers on the compound a useful property with respect to the treatment or imaging or diagnosis of diseases or other conditions or states which involve undesirable neovasculature formation. Such diseases or other conditions or states are described in more detail below. In particular, the further moiety is one which is useful in killing
15 or imaging neovasculature associated with the growth of a tumour. Preferably, the further moiety is one which is able to kill the endothelial cells to which the compound is targeted.

- In a preferred embodiment of the invention the further moiety is directly or
20 indirectly cytotoxic. In particular the further moiety is preferably directly or indirectly toxic to cells in neovasculature or cells which are in close proximity to and associated with neovasculature.

- By "directly cytotoxic" we include the meaning that the moiety is one which
25 on its own is cytotoxic. By "indirectly cytotoxic" we include the meaning that the moiety is one which, although is not itself cytotoxic, can induce

cytotoxicity, for example by its action on a further molecule or by further action on it.

In one embodiment the cytotoxic moiety is a cytotoxic chemotherapeutic agent.

5 Cytotoxic chemotherapeutic agents are well known in the art.

Cytotoxic chemotherapeutic agents, such as anticancer agents, include: alkylating agents including nitrogen mustards such as mechlorethamine (HN₂), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil; 10 ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa; alkyl sulphonates such as busulfan; nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and streptozocin (streptozotocin); and triazenes such as decarbazine (DTIC; dimethyltriazenoimidazole-carboxamide); Antimetabolites including folic acid analogues such as 15 methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycoformycin). Natural Products including vinca alkaloids 20 such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorubicin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin C); enzymes such as L-asparaginase; and biological response modifiers such as interferon alphenomes. Miscellaneous agents 25 including platinum coordination complexes such as cisplatin (*cis*-DDP) and carboplatin; anthracenedione such as mitoxantrone and anthracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-

methyldiazine, MIH); and adrenocortical suppressant such as mitotane (*o,p'*-DDD) and aminoglutethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen.

- 5 Various of these agents have previously been attached to antibodies and other target site-delivery agents, and so compounds of the invention comprising these agents may readily be made by the person skilled in the art. For example, carbodiimide conjugation (Bauminger & Wilchek (1980) *Methods Enzymol.* 70, 151-159; incorporated herein by reference) may be used to conjugate a
10 variety of agents, including doxorubicin, to antibodies or peptides.

Carbodiimides comprise a group of compounds that have the general formula $R-N=C=N-R'$, where R and R' can be aliphatic or aromatic, and are used for synthesis of peptide bonds. The preparative procedure is simple, relatively
15 fast, and is carried out under mild conditions. Carbodiimide compounds attack carboxylic groups to change them into reactive sites for free amino groups.

The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is particularly useful for conjugating a functional moiety
20 to a binding moiety and may be used to conjugate doxorubicin to tumor homing peptides. The conjugation of doxorubicin and a binding moiety requires the presence of an amino group, which is provided by doxorubicin, and a carboxyl group, which is provided by the binding moiety such as an antibody or peptide.

25

In addition to using carbodiimides for the direct formation of peptide bonds, EDC also can be used to prepare active esters such as N-hydroxysuccinimide

(NHS) ester. The NHS ester, which binds only to amino groups, then can be used to induce the formation of an amide bond with the single amino group of the doxorubicin. The use of EDC and NHS in combination is commonly used for conjugation in order to increase yield of conjugate formation (Bauminger &
5 Wilchek, *supra*, 1980).

Other methods for conjugating a functional moiety to a binding moiety also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can glutaraldehyde cross-
10 linking. However, it is recognised that, regardless of which method of producing a conjugate of the invention is selected, a determination must be made that the binding moiety maintains its targeting ability and that the functional moiety maintains its relevant function.

15 In a further embodiment of the invention, the cytotoxic moiety is a cytotoxic peptide or polypeptide moiety by which we include any moiety which leads to cell death. Cytotoxic peptide and polypeptide moieties are well known in the art and include, for example, ricin, abrin, *Pseudomonas* exotoxin, tissue factor and the like. Methods for linking them to targeting moieties such as antibodies
20 are also known in the art. The use of ricin as a cytotoxic agent is described in Burrows & Thorpe (1993) *Proc. Natl. Acad. Sci. USA* 90, 8996-9000, incorporated herein by reference, and the use of tissue factor, which leads to localised blood clotting and infarction of a tumour, has been described by Ran
25 *et al* (1998) *Cancer Res.* 58, 4646-4653 and Huang *et al* (1997) *Science* 275, 547-550. Tsai *et al* (1995) *Dis. Colon Rectum* 38, 1067-1074 describes the abrin A chain conjugated to a monoclonal antibody and is incorporated herein by reference. Other ribosome inactivating proteins are described as cytotoxic

agents in WO 96/06641. *Pseudomonas* exotoxin may also be used as the cytotoxic polypeptide moiety (see, for example, Aiello *et al* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10457-10461; incorporated herein by reference).

- 5 Certain cytokines, such as TNF α and IL-2, may also be useful as cytotoxic agents.

Certain radioactive atoms may also be cytotoxic if delivered in sufficient doses. Thus, the cytotoxic moiety may comprise a radioactive atom which, in use,
10 delivers a sufficient quantity of radioactivity to the target site so as to be cytotoxic. Suitable radioactive atoms include phosphorus-32, iodine-125, iodine-131, indium-111, rhenium-186, rhenium-188 or yttrium-90, or any other isotope which emits enough energy to destroy neighbouring cells, organelles or nucleic acid. Preferably, the isotopes and density of radioactive atoms in the
15 compound of the invention are such that a dose of more than 4000 cGy (preferably at least 6000, 8000 or 10000 cGy) is delivered to the target site and, preferably, to the cells at the target site and their organelles, particularly the nucleus.

- 20 The radioactive atom may be attached to the binding moiety in known ways. For example EDTA or another chelating agent may be attached to the binding moiety and used to attach ^{111}In or ^{90}Y . Tyrosine residues may be labelled with ^{125}I or ^{131}I .

- 25 The cytotoxic moiety may be a suitable indirectly cytotoxic polypeptide. In a particularly preferred embodiment, the indirectly cytotoxic polypeptide is a polypeptide which has enzymatic activity and can convert a relatively non-

toxic prodrug into a cytotoxic drug. When the targeting moiety is an antibody this type of system is often referred to as ADEPT (Antibody-Directed Enzyme Prodrug Therapy). The system requires that the targeting moiety locates the enzymatic portion to the desired site in the body of the patient (ie the site
5 expressing ECSM1 or ECSM4, such as new vascular tissue associated with a tumour) and after allowing time for the enzyme to localise at the site, administering a prodrug which is a substrate for the enzyme, the end product of the catalysis being a cytotoxic compound. The object of the approach is to maximise the concentration of drug at the desired site and to minimise the
10 concentration of drug in normal tissues (see Senter, P.D. *et al* (1988) "Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate" *Proc. Natl. Acad. Sci. USA* **85**, 4842-4846; Bagshawe (1987) *Br. J. Cancer* **56**, 531-2; and Bagshawe, K.D. *et al* (1988) "A cytotoxic agent can be generated selectively at cancer sites" *Br. J. Cancer.* **58**, 700-703.)

15

Clearly, any ECSM1 or ECSM4 binding moiety may be used in place of an anti-ECSM1 or anti-ECSM4 antibody in this type of directed enzyme prodrug therapy system.

20 The enzyme and prodrug of the system using an ECSM1 or ECSM4 targeted enzyme as described herein may be any of those previously proposed. The cytotoxic substance may be any existing anti-cancer drug such as an alkylating agent; an agent which intercalates in DNA; an agent which inhibits any key enzymes such as dihydrofolate reductase, thymidine synthetase, ribonucleotide
25 reductase, nucleoside kinases or topoisomerase; or an agent which effects cell death by interacting with any other cellular constituent. Etoposide is an example of a topoisomerase inhibitor.

Reported prodrug systems include: a phenol mustard prodrug activated by an *E. coli* β -glucuronidase (Wang *et al*, 1992 and Roffler *et al*, 1991); a doxorubicin prodrug activated by a human β -glucuronidase (Bosslet *et al*,
5 1994); further doxorubicin prodrugs activated by coffee bean α -galactosidase (Azoulay *et al*, 1995); daunorubicin prodrugs, activated by coffee bean α -D-galactosidase (Gesson *et al*, 1994); a 5-fluorouridine prodrug activated by an *E. coli* β -D-galactosidase (Abraham *et al*, 1994); and methotrexate prodrugs (eg methotrexate-alanine) activated by carboxypeptidase A (Kuefner *et al*, 1990,
10 Vitols *et al*, 1992 and Vitols *et al*, 1995). These and others are included in the following table.

Enzyme	Prodrug
Carboxypeptidase G2	Derivatives of L-glutamic acid and benzoic acid mustards, aniline mustards, phenol mustards and phenylenediamine mustards; fluorinated derivatives of these
Alkaline phosphatase	Etoposide phosphate Mitomycin phosphate
Beta-glucuronidase	<i>p</i> -Hydroxyaniline mustard-glucuronide Epirubicin-glucuronide
Penicillin-V-amidase	Adriamycin-N phenoxyacetyl
Penicillin-G-amidase	N-(4'-hydroxyphenyl acetyl) palytoxin Doxorubicin and melphalan
Beta-lactamase	Nitrogen mustard-cephalosporin <i>p</i> -phenylenediamine; doxorubicin derivatives; vinblastine derivative-cephalosporin, cephalosporin mustard; a taxol derivative
Beta-glucosidase	Cyanophenylmethyl-beta-D-glucopyranosiduronic acid
Nitroreductase	5-(Azaridin-1-yl)-2,4-dinitrobenzamide
Cytosine deaminase	5-Fluorocytosine
Carboxypeptidase A	Methotrexate-alanine

(This table is adapted from Bagshawe (1995) *Drug Dev. Res.* 34, 220-230, from which full references for these various systems may be obtained; the taxol derivative is described in Rodrigues, M.L. *et al* (1995) *Chemistry & Biology* 2, 223).

5

Suitable enzymes for forming part of the enzymatic portion of the invention include: exopeptidases, such as carboxypeptidases G, G1 and G2 (for glutamylated mustard prodrugs), carboxypeptidases A and B (for MTX-based prodrugs) and aminopeptidases (for 2- α -aminocyl MTC prodrugs);
10 endopeptidases, such as eg thrombolysin (for thrombin prodrugs); hydrolases, such as phosphatases (eg alkaline phosphatase) or sulphatases (eg aryl sulphatases) (for phosphorylated or sulphated prodrugs); amidases, such as penicillin amidases and arylacyl amidase; lactamases, such as β -lactamases; glycosidases, such as β -glucuronidase (for β -glucuronomide anthracyclines), α -
15 galactosidase (for amygdalin) and β -galactosidase (for β -galactose anthracycline); deaminases, such as cytosine deaminase (for 5FC); kinases, such as urokinase and thymidine kinase (for gancyclovir); reductases, such as nitroreductase (for CB1954 and analogues), azoreductase (for azobenzene mustards) and DT-diaphorase (for CB1954); oxidases, such as glucose oxidase
20 (for glucose), xanthine oxidase (for xanthine) and lactoperoxidase; DL-racemases, catalytic antibodies and cyclodextrins.

The prodrug is relatively non-toxic compared to the cytotoxic drug. Typically, it has less than 10% of the toxicity, preferably less than 1% of the toxicity as
25 measured in a suitable *in vitro* cytotoxicity test.

It is likely that the moiety which is able to convert a prodrug to a cytotoxic drug will be active in isolation from the rest of the compound but it is necessary only for it to be active when (a) it is in combination with the rest of the compound and (b) the compound is attached to, adjacent to or internalised in target cells.

When each moiety of the compound is a polypeptide, the two portions may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al* (1979) *Anal. Biochem.* 100, 100-108. For example, the ECSM1 or ECSM4 binding moiety may be enriched with thiol groups and the further moiety reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

Alternatively, the compound may be produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two moieties of the compound of the invention either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the compound. Conceivably, the two portions of the compound may overlap wholly or partly.

The DNA is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention.

The invention also provides a kit of parts (or a therapeutic system) comprising (1) a compound of the invention wherein the further moiety which is able to convert a relatively non-toxic prodrug into a cytotoxic drug and (2) a relatively non-toxic prodrug. The kit of parts may comprise any of the compounds of the invention and appropriate prodrugs as herein disclosed.

The invention also provides a kit of parts (or a therapeutic system) comprising (1) a compound of the invention wherein the further moiety is able to bind selectively to a directly or indirectly cytotoxic moiety or to a readily detectable moiety and (2) any one of a directly or indirectly cytotoxic or a readily detectable moiety to which the further moiety of the compound is able to bind.

The cytotoxic moiety may be a radiosensitizer. Radiosensitizers include fluoropyrimidines, thymidine analogues, hydroxyurea, gemcitabine, fludarabine, nicotinamide, halogenated pyrimidines, 3-aminobenzamide, 3-aminobenzodiamide, etanixadole, pimonidazole and misonidazole (see, for example, McGinn *et al* (1996) *J. Natl. Cancer Inst.* **88**, 1193-11203; Shewach & Lawrence (1996) *Invest. New Drugs* **14**, 257-263; Horsman (1995) *Acta Oncol.* **34**, 571-587; Shenoy & Singh (1992) *Clin. Invest.* **10**, 533-551; Mitchell *et al* (1989) *Int. J. Radiat. Biol.* **56**, 827-836; Iliakis & Kurtzman (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 1235-1241; Brown (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 987-993; Brown (1985) *Cancer* **55**, 2222-2228).

Also, delivery of genes into cells can radiosensitise them, for example delivery of the p53 gene or cyclin D (Lang *et al* (1998) *J. Neurosurg.* **89**, 125-132; Coco Martin *et al* (1999) *Cancer Res.* **59**, 1134-1140).

The further moiety may be one which becomes cytotoxic, or releases a cytotoxic moiety, upon irradiation. For example, the boron-10 isotope, when appropriately irradiated, releases α particles which are cytotoxic (see for example, US 4, 348, 376 to Goldenberg; Primus *et al* (1996) *Bioconjug. Chem.* 7, 532-535).

Similarly, the cytotoxic moiety may be one which is useful in photodynamic therapy such as photofrin (see, for example, Dougherty *et al* (1998) *J. Natl. Cancer Inst.* 90, 889-905).

10

The further moiety may comprise a nucleic acid molecule which is directly or indirectly cytotoxic. For example, the nucleic acid molecule may be an antisense oligonucleotide which, upon localisation at the target site is able to enter cells and lead to their death. The oligonucleotide, therefore, may be one which prevents expression of an essential gene, or one which leads to a change in gene expression which causes apoptosis.

Examples of suitable oligonucleotides include those directed at bcl-2 (Ziegler *et al* (1997) *J. Natl. Cancer Inst.* 89, 1027-1036), and DNA polymerase α and topoisomerase II α (Lee *et al* (1996) *Anticancer Res.* 16, 1805-1811).

Peptide nucleic acids may be useful in place of conventional nucleic acids (see Knudsen & Nielsen (1997) *Anticancer Drugs* 8, 113-118).

In a further embodiment, the binding moiety may be comprised in a delivery vehicle for delivering nucleic acid to the target. The delivery vehicle may be any suitable delivery vehicle. It may, for example, be a liposome containing

nucleic acid, or it may be a virus or virus-like particle which is able to deliver nucleic acid. In these cases, the moiety which selectively binds to ECSM1 or ECSM4 is typically present on the surface of the delivery vehicle. For example, the moiety which selectively binds to ECSM1 or ECSM4, such as a
5 suitable antibody fragment, may be present in the outer surface of a liposome and the nucleic acid to be delivered may be present in the interior of the liposome. As another example, a viral vector, such as a retroviral or adenoviral vector, is engineered so that the moiety which selectively binds to ECSM1 or ECSM4 is attached to or located in the surface of the viral particle thus
10 enabling the viral particle to be targeted to the desired site. Targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* 2, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre
15 protein. Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into preexisting viral *env* genes (see Miller & Vile (1995) *Faseb J.* 9, 190-199 for a review of this and other targeted vectors for gene therapy).

20 Immunoliposomes (antibody-directed liposomes) may be used in which the moiety which selectively binds to ECSM1 or ECSM4 is an antibody. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-
25 288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the anti-ECSM1 or -ECSM4 antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other

genetic construct for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, 5 entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The 10 immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

The nucleic acid delivered to the target site may be any suitable DNA which 15 leads, directly or indirectly, to cytotoxicity. For example, the nucleic acid may encode a ribozyme which is cytotoxic to the cell, or it may encode an enzyme which is able to convert a substantially non-toxic prodrug into a cytotoxic drug (this latter system is sometime called GDEPT: Gene Directed Enzyme Prodrug Therapy).

20

Ribozymes which may be encoded in the nucleic acid to be delivered to the target are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNase P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" 25 US 5,149,796; Cech *et al* "RNA ribozyme restriction endoribonucleases and methods", US 5,116,742; Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endonucleases and methods", US 5,093,246; and

Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference. Suitable targets for ribozymes include transcription factors such as c-fos and c-myc, and bcl-2. Durai *et al* (1997) *Anticancer Res.* 17, 3307-3312 describes a
5 hammerhead ribozyme against bcl-2.

EP 0 415 731 describes the GDEPT system. Similar considerations concerning the choice of enzyme and prodrug apply to the GDEPT system as to the
10 ADEPT system described above.

The nucleic acid delivered to the target site may encode a directly cytotoxic polypeptide.

15 Alternatively, the further portion may comprise a polypeptide or a polynucleotide encoding a polypeptide which is not either directly or indirectly cytotoxic but is of therapeutic benefit. Examples of such polypeptides include anti-proliferative or anti-inflammatory cytokines which could be of benefit in atherosclerosis, and anti-proliferative, immunomodulatory or factors
20 influencing blood clotting may be of benefit in treating cancer.

The further moiety may usefully be an inhibitor of angiogenesis such as the peptides angiostatin or endostatin. The further moiety may also usefully be an enzyme which converts a precursor polypeptide to angiostatin or endostatin.
25 Human matrix metallo-proteases such as macrophage elastase, gelatinase and stromolysin convert plasminogen to angiostatin (Cornelius *et al* (1998) *J. Immunol.* 161, 6845-6852). Plasminogen is a precursor of angiostatin.

In a further embodiment of the invention, the further moiety comprised in the compound of the invention is a readily detectable moiety.

- 5 By a "readily detectable moiety" we include the meaning that the moiety is one which, when located at the target site following administration of the compound of the invention into a patient, may be detected, typically non-invasively from outside the body and the site of the target located. Thus, the compounds of this embodiment of the invention are useful in imaging and
10 diagnosis.

Typically, the readily detectable moiety is or comprises a radioactive atom which is useful in imaging. Suitable radioactive atoms include technetium-99m or iodine-123 for scintigraphic studies. Other readily detectable moieties
15 include, for example, spin labels for magnetic resonance imaging (MRI) such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron. Clearly, the compound of the invention must have sufficient of the appropriate atomic isotopes in order for the molecule to be readily detectable.

20

The radio- or other labels may be incorporated in the compound of the invention in known ways. For example, if the binding moiety is a polypeptide it may be biosynthesised or may be synthesised by chemical amino acid synthesis using suitable amino acid precursors involving, for example,
25 fluorine-19 in place of hydrogen. Labels such as ^{99m}Tc , ^{123}I , ^{186}Rh , ^{188}Rh and ^{111}In can, for example, be attached *via* cysteine residues in the binding moiety. Yttrium-90 can be attached *via* a lysine residue. The IODOGEN method

(Fraker *et al* (1978) *Biochem. Biophys. Res. Comm.* 80, 49-57) can be used to incorporate iodine-123. Reference ("Monoclonal Antibodies in Immunoscintigraphy", J-F Chatal, CRC Press, 1989) describes other methods in detail.

5

In a further preferred embodiment of the invention the further moiety is able to bind selectively to a directly or indirectly cytotoxic moiety or to a readily detectable moiety. Thus, in this embodiment, the further moiety may be any moiety which binds to a further compound or component which is cytotoxic or readily detectable.

10

The further moiety may, therefore be an antibody which selectively binds to the further compound or component, or it may be some other binding moiety such as streptavidin or biotin or the like. The following examples illustrate the types of molecules that are included in the invention; other such molecules are readily apparent from the teachings herein.

15

A bispecific antibody wherein one binding site comprises the moiety which selectively binds to ECSM1 or ECSM4 and the second binding site comprises a moiety which binds to, for example, an enzyme which is able to convert a substantially non-toxic prodrug to a cytotoxic drug.

20

A compound, such as an antibody which selectively binds to ECSM1 or ECSM4, to which is bound biotin. Avidin or streptavidin which has been labelled with a readily detectable label may be used in conjunction with the biotin labelled antibody in a two-phase imaging system wherein the biotin labelled antibody is first localised to the target site in the patient, and then the

25

labelled avidin or streptavidin is administered to the patient. Bispecific antibodies and biotin/streptavidin (avidin) systems are reviewed by Rosebrough (1996) *Q J Nucl. Med.* 40, 234-251.

- 5 In a preferred embodiment of the invention, the moiety which selectively binds to ECSM1 or ECSM4 and the further moiety are polypeptides which are fused.

The compounds of the first and second aspects of the invention are useful in treating, imaging or diagnosing disease, particularly diseases in which there
10 may be undesirable neovasculature formation, as described in more detail below.

In a preferred embodiment of the first and second aspects of the invention, the compounds are suitable for use in medicine.

15

A third aspect of the invention provides a nucleic acid molecule encoding a compound of either the first or second aspects of the invention wherein the selective binding moiety and the further moiety are polypeptides which are fused.

20

Methods of linking polynucleotides are described in more detail below.

A fourth aspect of the invention provides a pharmaceutical composition comprising a compound according to the invention and a pharmaceutically
25 acceptable carrier. The compound of the invention includes those described in the first, second and third aspects. The invention also includes pharmaceutical composition comprising any of an antibody, polypeptide, peptide,

polynucleotide, expression vector or other agent which may be delivered to an individual as described below and a pharmaceutically acceptable carrier.

By "pharmaceutically acceptable" is included that the formulation is sterile and
5 pyrogen free. Suitable pharmaceutical carriers are well known in the art of pharmacy.

The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof.
10 Typically, the carriers will be water or saline which will be sterile and pyrogen free; however, other acceptable carriers may be used.

Typically the pharmaceutical compositions or formulations of the invention are for parenteral administration, more particularly for intravenous administration.
15

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions
20 which may include suspending agents and thickening agents.

A fifth aspect of the invention provides a method of imaging vascular endothelium in the body of an individual the method comprising administering to the individual an effective amount of a compound according to either of the
25 first or second aspects of the invention wherein the further moiety is a readily detectable moiety.

Typically the vascular endothelium is associated with angiogenesis.

As discussed above in relation to the first and second aspects of the invention, the moiety of the compound which selectively binds ECSM4 or ECSM1 may
5 be an antibody. Preferred antibodies are as outlined above.

In a preferred embodiment of this aspect of the invention, the method of imaging the vascular endothelium in an individual comprises the further step of detecting the location of the compound in the individual.

10

Detecting the compound or antibody can be achieved using methods well known in the art of clinical imaging and diagnostics. The specific method required will depend on the type of detectable label attached to the compound or antibody. For example, radioactive atoms may be detected using
15 autoradiography or in some cases by magnetic resonance imaging (MRI) as described above.

Imaging the vascular endothelium in the body is useful because it can provide information about the health of the body. It is particularly useful when the
20 vascular endothelium is diseased, or is proliferating due to a cancerous growth. Imaging cancer in a patient is especially useful, because it can be used to determine the size of a tumour and whether it is responding to treatment. Since metastatic disease involves new blood vessel formation, the method is useful in assessing whether metastasis has occurred.

25

Hence, in a preferred embodiment of the fifth aspect of the invention, the vascular endothelium is neovasculature, such as that produced in cancer.

A sixth aspect of the invention provides a method of diagnosing or prognosing in an individual a condition which involves the vascular endothelium the method comprising administering to the individual an effective amount of a compound according to either of the first or second aspects of the invention
5 wherein the further moiety is a readily detectable moiety.

The condition may be one which involves aberrant or excessive growth of vascular endothelium, such as cancer, arteriosclerosis, restenosis, diabetic
10 retinopathy, arthritis, psoriasis, endometriosis, menorrhagia, haemangiomas and venous malformations.

As discussed in relation to the first and second aspects of the invention, the compound may comprise an antibody. The antibody may be any antibody
15 which selectively binds the polypeptide ECSM1 or ECSM4 as required. Preferred antibodies which bind the polypeptide ECSM4 are as outlined above.

The method may be one which is an aid to diagnosis.

20 In a preferred embodiment of this aspect of the invention, the method of diagnosing, or aiding diagnosis of, a condition involving the vascular endothelium in an individual comprises the further step of detecting the location of the compound in the individual. Preferably the endothelium is in neovasculature; ie, angiogenic vasculature.

25

The function of ECSM4 or ECSM1 may not be to promote proliferation of vascular endothelial cells. Therefore the level of expression of these

polypeptides within an endothelial cell may not be informative about the health of the vascular endothelium. However, the location of expression of the polypeptides may be informative, as they represent the growth of blood vessels. Abnormal cell proliferation such as cancer may be diagnosed by the
5 detection of new vasculature.

A seventh aspect of the invention provides a method of treating an individual in need of treatment, the method comprising administering to the individual an effective amount of a compound according to the first or second aspects of the
10 invention wherein the further moiety is a cytotoxic or therapeutic moiety.

In one embodiment of this aspect, the patient in need of treatment has a proliferative disease or a condition involving the vascular endothelium.

15 A number of diseases and conditions involve undesirable neovasculature formation. Neovasculature formation is associated with cancer, psoriasis, atherosclerosis, menorrhagia, arthritis (both inflammatory and rheumatoid), macular degeneration, Paget's disease, retinopathy and its vascular complications (including proliferative and of prematurity, and diabetic), benign
20 vascular proliferations and fibroses.

By cancer is included Kaposi's sarcoma, leukaemia, lymphoma, myeloma, solid carcinomas (both primary and secondary (metastasis), vascular tumours including haemangioma (both capillary and juvenile (infantile)),
25 haemangiomatosis and haemangioblastoma.

Thus, the invention comprises a method of treating a patient who has a disease in which angiogenesis contributes to pathology the method comprising the step of administering to the patient an effective amount of a compound of the first or second aspect of the invention wherein the further moiety of the compound
5 is one which either directly or indirectly is of therapeutic benefit to the patient.

Typically, the disease is associated with undesirable neovasculature formation and the treatment reduces this to a useful extent.

10 The tumours that may be treated by the methods of the invention include any tumours which are associated with new blood vessel production.

The term "tumour" is to be understood as referring to all forms of neoplastic cell growth, including tumours of the lung, liver, blood cells, skin, pancreas, stomach, colon, prostate, uterus, breast, lymph glands and bladder. Solid
15 tumours are especially suitable. However, blood cancers, including leukaemias and lymphomas are now also believed to involve new blood vessel formation and may be treated by the methods of the invention.

20 Typically in the above-mentioned methods of treatment, the further moiety is one which destroys or slows or reverses the growth of the neovasculature.

It will readily be appreciated that, depending on the particular compound used in imaging, diagnosis or treatment, the timing of administration may vary and
25 the number of other components used in therapeutic systems disclosed herein may vary.

For example, in the case where the compound of the invention comprises a readily detectable moiety or a directly cytotoxic moiety, it may be that only the compound, in a suitable formulation, is administered to the patient. Of course, other agents such as immunosuppressive agents and the like may be
5 administered.

In respect of compounds which are detectably labelled, imaging takes place once the compound has localised at the target site.

10 However, if the compound is one which requires a further component in order to be useful for treatment, imaging or diagnosis, the compound of the invention may be administered and allowed to localise at the target site, and then the further component administered at a suitable time thereafter.

15 For example, in respect of the ADEPT and ADEPT-like systems above, the binding moiety-enzyme moiety compound is administered and localises to the target site. Once this is done, the prodrug is administered.

Similarly, for example, in respect of the compounds wherein the further moiety
20 comprised in the compound is one which binds a further component, the compound may be administered first and allowed to localise at the target site, and subsequently the further component is administered.

Thus, in one embodiment a biotin-labelled anti-ECSM1 or -ECSM4 antibody is
25 administered to the patient and, after a suitable period of time, detectably labelled streptavidin is administered. Once the streptavidin has localised to the sites where the antibody has localised (ie the target sites) imaging takes place.

Where the compound whose moiety which selectively binds is an antibody, the antibody may be any antibody which selectively binds the polypeptide ECSM1 or ECSM4 as required. Preferred antibodies are as outlined in the
5 first and second aspects of the invention.

It is believed that the compounds of the invention wherein the further moiety is a readily detectable moiety may be useful in determining the angiogenic status of tumours or other disease states in which angiogenesis contributes to
10 pathology. This may be an important factor influencing the nature and outcome of future therapy.

An eighth aspect of the invention provides a method of introducing genetic material selectively into vascular endothelial cells the method comprising
15 contacting the cells with a compound according to either of the first or second aspects of the invention as described above wherein the further moiety is a nucleic acid.

The vascular endothelial cells may be any vascular endothelial cells such as
20 those in tissue culture or in a living organism. It is preferred if the cells are in a living organism. It is further preferred if the organism is a human. It is still more preferred if the vascular endothelial cells are those in neovasculature, ie they are angiogenic endothelial cells.

25 Preferably, the binding moiety is an antibody. The antibody may be any antibody which selectively binds the polypeptide ECSM1 or ECSM4 as required. Preferably, the antibody is one as defined above in relation to the

first or second aspects of the invention. Typically, the binding moiety is comprised in a delivery vehicle and preferably, the delivery vehicle is a liposome, as described in further detail above. In this embodiment, the further moiety is nucleic acid and is comprised within the liposome, also as described
5 above. Typically, the method is used in gene therapy, and the genetic material is therapeutically useful. Therapeutically useful genetic material includes that which encodes a therapeutic protein.

A ninth aspect of the invention provides a use of a compound according to
10 either of the first or second aspects of the invention wherein the further moiety is a readily detectable label in the manufacture of a diagnostic or prognostic agent for a condition which involves the vascular endothelium.

As discussed above, the compound may comprise an antibody as the moiety
15 which selectively binds. The antibody may be any antibody which selectively binds the polypeptide ECSM1 or ECSM4 as required.

A tenth aspect of the invention provides a use of a compound according to either of the first or second aspects of the invention wherein the further moiety
20 is a cytotoxic or therapeutic moiety in the manufacture of a medicament for treating a condition involving the vascular endothelium.

Conditions which involve the vascular endothelium are described above.

25 As described above, the compound may comprise an antibody as the moiety which selectively binds. The antibody may be any suitable antibody which selectively binds the polypeptide ECSM1 or ECSM4 as required.

An eleventh aspect of the invention provides a polypeptide comprising or consisting of a fragment or variant or fusion of the ECSM4 polypeptide or a fusion of said fragment or variant provided that it is not a polypeptide
5 consisting of the amino acid sequence given between residues 49 and 466 of Figure 4.

The ECSM4 polypeptide includes a polypeptide comprising or consisting of the amino acid sequence given in Figure 4 or Figure 5 or Figure 7 or Figure 12
10 or Figure 13 or the polypeptide encoded by the nucleotide sequence of either Figure 4 between positions 1 and 1395 or Figure 5 between positions 2 and 948 or Figure 7 or Figure 12 or Figure 13 is that of the ECSM4 polypeptide. Preferably, the ECSM4 polypeptide of the invention comprises but does not consist of the amino acid sequence given in Figure 4.

15

Preferably, the ECSM4 polypeptide of the invention does not consist of any of the amino acid sequences represented by SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293,
20 or any of the amino acid sequences encoded by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00/53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of WO 99/11293.

A twelfth aspect of the invention provides a polypeptide comprising or
25 consisting of the ECSM1 polypeptide or a fragment or variant or fusion thereof or a fusion of said fragment or variant.

The ECSM1 polypeptide includes a polypeptide comprising or consisting of the amino acid sequence given in Figure 2. Preferably, the ECSM1 polypeptide or fragment is not a polypeptide whose sequence is given in SEQ ID No 120 of WO 99/06423 or which is encoded by SEQ ID No 32 of WO 99/06423 or encoded by the nucleic acid of ATCC deposit No 209145 made on
5 July 17 1997 for the purposes of WO 99/06423.

The invention includes peptides which are derived from the ECSM4 or ECSM1 polypeptides. These peptides may be considered "fragments" of the ECSM4 or
10 ECSM1 polypeptides but may be produced by *de novo* synthesis or by fragmentation of the polypeptide.

"Fragments" of the ECSM4 or ECSM1 polypeptide include polypeptides which comprise at least five consecutive amino acids of the ECSM4 or ECSM1
15 polypeptide. Preferably, a fragment of the polypeptide comprises an amino acid sequence which is useful, for example, a fragment which retains activity of the polypeptide, or a fragment for use in a binding assay or is useful as a peptide for producing an antibody which is specific for the ECSM4 or ECSM1 polypeptide. An activity of the ECSM4 polypeptide may be in endothelial cell
20 repulsive guidance. Repulsive guidance may be tested *in vivo* by constructing appropriate transgenic or knock-out animal models, for example mice or zebrafish. It may also be tested *in vivo* on cell migration assays such as Boyden chamber or video microscopy. Typically, the fragments have at least 8 consecutive amino acids, preferably at least 10, more preferably at least 12 or
25 15 or 20 or 30 or 40 or 50 consecutive amino acids of the ECSM4 or ECSM1 polypeptide. Preferably, fragments of the ECSM4 polypeptide comprise but do not consist of the amino acid sequence given in Figure 4 or Figure 5 or Figure

7 or Figure 12 or Figure 13. Preferably, fragments of the ECSM4 polypeptide comprise but do not consist of any of the amino acid sequences represented by SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or
 5 SEQ ID No 86 of WO 99/11293, or any of the amino acid sequences encoded by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00 53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of WO 99/11293.

- 10 Typically, the fragments of ECSM4 polypeptide are ones which have portions of the amino acid sequence shown in Figure 4 or Figure 12.

Typically, the fragments of ECSM1 polypeptide are ones which have portions of the amino acid sequence shown in Figure 2.

15

In a preferred embodiment of the thirteenth aspect of the invention, a fragment of the ECSM4 polypeptide is a fragment which has the sequence LSQSPGAVPQALVAWRA, DSVLTPEEVALCLEL, TYGYISVPTA, KGGVLLCPPRPCLTPT, WLADTW, WLADTWRSTSGSRD,
 20 SPPTTYGYIS, GSLANGWGSASEDNAASARASLVSSSDGSFLAD or FARALAVAVD or has a sequence of at least 5 or 8 or 10 residues of any of these sequences. These peptides correspond to amino acids 165-181, 274-288, 311-320, 336-351, 8-13, 8-21, 307-316, 355-387 and 390-399 respectively of the human ECSM4 polypeptide shown in Figure 4. Peptides WLADTW,
 25 WLADTWRSTSGSRD, SPPTTYGYIS, GSLANGWGSASEDNAASARASLVSSSDGSFLAD and FARALAVAVD represent conserved regions between the mouse and human homologues of the

ECSM4 polypeptide, and between the ECSM4 polypeptide and the mouse dutt1 protein. The peptides LSQSPGAVPQALVAWRA, DSVLTPEEVALCLEL, TYGYISVPTA and KGGVLLCPPRPCLTPT may be useful in raising antibodies.

5

Preferred peptides are peptides of at least 5 or 8 or 10 or 12 or 15 or 20 consecutive amino acid residues from these conserved sequences. Peptides of ECSM4 which affect cell migration and/or growth and/or vascular development are particularly preferred. They can be identified in suitable
10 screening systems.

In a further preferred embodiment of this aspect of the invention, a fragment of the ECSM4 polypeptide is a fragment which has the sequence GGDSLLGGRGSL, LLQPPARGHAHDGQALSTD, EPQDYTEPVE,
15 TAPGGQGAPWAEE or ERATQEPSEHGP or has a sequence of at least 5 or 8 or 10 residues of any of these sequences. These peptides correspond to regions of the human ECSM4 polypeptide (located at residues 4-16, 91-109, 227-236, 288-300 and 444-455 respectively in the sequence given in Figure 12) which are not, or are poorly, conserved in the mouse homologue (see Figure
20 14). As described below, such peptides may be particularly useful in raising antibodies to the human ECSM4 polypeptide.

According to the transmembrane domain predicting software program called PRED-TMR (available at the internet site <http://www.biophys.biol.uoa.gr>) and
25 an amino acid sequence alignment with the human protein Robo1 (whose transmembrane region is known), residues 1-467 as shown in Figure 12 are likely to be extracellular, and in addition to being extracellularly exposed, may

include the binding site of the natural ligand. Hence fragments of ECSM4 which include or consist of a sequence within the extracellular domain of residues 1-467 of Figure 12 may represent useful fragments for raising antibodies selective for cells expressing ECSM4 on their surface and which
5 may also be useful in modulating the activity of the polypeptide ECSM4.

Hence, preferred fragments of the ECSM4 polypeptide are those fragments of the polypeptide sequence of Figure 12 which comprise at least 1, 3 or 5, amino acid residues which are not conserved when compared to the mouse ECSM4
10 (as shown in Figure 13). More preferably at least 7, 9, 11 or 13 amino acid residues in the fragment are not conserved between human ECSM4 and mouse ECSM4, and still more preferably at least 15, 17, 19 or 21 residues of the fragment are not conserved between human ECSM4 and mouse ECSM4. The sequence of such fragments may be determined from the alignment of the
15 human and mouse amino acid sequences shown in Figure 14.

It will be appreciated that fragments of the ECSM4 or ECSM1 polypeptide of the invention are particularly useful when fused to other polypeptides, such as glutathione-S-transferase (GST), green fluorescent protein (GFP), vesicular
20 stomatitis virus glycoprotein (VSVG) or keyhole limpet haemocyanin (KLH). Fusions of the polypeptide, or fusions of fragments or variants of the polypeptide of the invention are included in the scope of the invention.

Other useful fragments of ECSM4 are those which are able to bind a ligand
25 selective for ECSM4. Suitable methods for identification of ligands such as peptides or other molecules which bind ECSM4 is discussed in more detail above. Such peptides or other ECSM4-binding molecules can be used to identify the amino acid sequences present in ECSM4 which are responsible for

ligand binding. Identification of those fragments of ECSM4 which, when isolated from the rest of the molecule, are still able to bind a ligand of ECSM4 can be achieved by means of a screen. Typically, such a screen will comprise contacting a ligand of ECSM4 with a test fragment of the ECSM4 polypeptide and determining if the test fragment binds the ligand. Fragments of ECSM4 are within the scope of the invention, and may be particularly useful in medicine. A fragment of ECSM4 which binds the natural ECSM4 ligand may neutralise the effect of the ligand and thereby affect endothelial cell migration, growth and/or vascular development. Hence, administration of fragments of ECSM4 may be useful in the treatment of diseases or conditions where endothelial cell migration, growth and/or vascular development need to be modulated. Examples of such diseases include cancer and arteriosclerosis.

A "fusion" of the ECSM4 or ECSM1 polypeptide or a fragment or variant thereof provides a molecule comprising a polypeptide of the invention and a further portion. It is preferred that the said further portion confers a desirable feature on the said molecule; for example, the portion may be useful in detecting or isolating the molecule, or promoting cellular uptake of the molecule. The portion may be, for example, a biotin moiety, a radioactive moiety, a fluorescent moiety, for example a small fluorophore or a green fluorescent protein (GFP) fluorophore, as well known to those skilled in the art. The moiety may be an immunogenic tag, for example a Myc tag, as known to those skilled in the art or may be a lipophilic molecule or polypeptide domain that is capable of promoting cellular uptake of the molecule or the interacting polypeptide, as known to those skilled in the art.

A "variant" of the ECSM4 or ECSM1 polypeptide includes natural variants, including allelic variants and naturally-occurring mutant forms and variants with insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the activity of the
5 said polypeptide. In the case of the ECSM4 polypeptide, as an endothelial specific homologue of the human roundabout 1 it may well be involved in endothelial cell repulsive guidance. In addition, polypeptides which are elongated as a result of an insertion or which are truncated due to deletion of a region are included in the scope of the invention. For example, deletion of
10 cytoplasmically-located regions may be useful in creation of "dominant negative" or "dominant positive" forms of the polypeptide. Similarly, deletion of a transmembrane region of the polypeptide may produce such forms.

By "conservative substitution" is intended combinations such as Gly, Ala; Val,
15 Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

By "non-conservative substitution" we include other substitutions, such as those where the substituted residue mimics a particular modification of the replaced residue, for example a phosphorylated tyrosine or serine may be
20 replaced by aspartate or glutamate due to the similarity of the aspartate or glutamate side chain to a phosphorylated residue (ie they carry a negative charge at neutral pH).

Further non-conservative substitutions which are included in the term
25 "variants" are point mutations which alter one, sometimes two, and usually no more than three amino acids. Such mutations are well known in the art of biochemistry and are usually designed to insert or remove a defined

characteristic of the polypeptide. Another type of non-conservative mutation is the alteration or addition of a residue to a cysteine or lysine residue which can then be used with maleimide or succinimide cross-linking reagents to covalently conjugate the polypeptide to another moiety. Non-glycosylated
5 proteins may be mutated to convert an asparagine to the recognition motif N-X-S/T for N-linked glycosylation. Such a modification may be useful to create a tag for purification of the polypeptide using Concanavalin A-linked beads.

Such variants may be made using the methods of protein engineering and site-
10 directed mutagenesis well known in the art.

Variants of the ECSM4 polypeptide include polypeptides comprising a sequence with at least 65% identity to the amino acid sequence given in Figure 4 or Figure 7 or Figure 12 or Figure 13, preferably at least 70% or 80% or 85%
15 or 90% identity to said sequence, and more preferably at least 95% or 98% identity to said amino acid sequence.

Variants of the ECSM1 polypeptide include polypeptides comprising a sequence with at least 65% identity to the amino acid sequence given in Figure
20 2, preferably at least 70% or 80% or 85% or 90% identity to said sequence, and more preferably at least 95% or 98% identity to said amino acid sequence.

Percent identity can be determined by, for example, the LALIGN program (Huang and Miller, *Adv. Appl. Math.* (1991) 12:337-357) at the Expasy facility
25 site (http://www.ch.embnet.org/software/LALIGN_form.html) using as parameters the global alignment option, scoring matrix BLOSUM62, opening gap penalty -14, extending gap penalty -4.

A thirteenth aspect of the invention provides a polynucleotide encoding the ECSM4 polypeptide of the invention, or the complement thereof or a polynucleotide which selectively hybridises to either of these which
5 polynucleotide is not any one of the clones corresponding to GenBank Accession No AK000805 or the ESTs whose GenBank Accession Nos are given in Table 11 or Table 12.

GenBank Accession No AK000805 corresponds to a cDNA sequence cloned in
10 the vector pME18SFL3. ESTs listed in Table 11 represent nucleotide sequences which can be assembled into the contig sequence shown in Figure 5. ESTs listed in Table 12 represent nucleotide sequences which can be assembled into the mouse nucleotide cluster sequence (Mm.27782) given in Figure 7.

15

Preferably, the polynucleotide of this aspect of the invention does not consist of any one of the nucleotide sequences represented by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00 53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of WO
20 99/11293, or their complement.

Also preferably, the polynucleotide of this aspect of the invention is not a polynucleotide which encodes a polypeptide consisting of the amino acid sequence represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ
25 ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293

Polynucleotides of the thirteenth aspect of the invention are described in more detail below.

A fourteenth aspect of the invention provides a polynucleotide encoding the
5 EC5M1 polypeptide or the complement thereof or a polynucleotide which
selectively hybridises to either of these, according to the twelfth aspect of the
invention provided that the polynucleotide is not one present in ATCC deposit
No 209145 or the clone corresponding to GenBank Accession No AC011526
or the ESTs whose GenBank Accession Nos are given in Table 10.

10

By "encoding a polypeptide according to the twelfth aspect of the invention"
we mean that the polynucleotide is one which encodes an EC5M1 polypeptide
of the invention and is not one which encodes a polypeptide whose sequence is
given in SEQ ID No 120 of WO 99/06423 or which is encoded by SEQ ID No
15 32 or by the nucleic acid included in the microbiological deposit corresponding
to American Type Culture Collection (ATCC) No. 209145 made on 17 July
1997.

ATCC deposit No 209145 comprises a pSport1 vector which includes a 765
20 base nucleotide sequence.

The polynucleotide sequence given in SEQ ID No 32 of WO 99/06423 is
similar to the nucleotide sequence shown in Figure 2. The sequence of SEQ ID
No 32 given in WO 99/06423 may be capable of encoding part of the EC5M1
25 polypeptide of the invention. Due to degeneracy of the genetic code however,
a polynucleotide sequence may encode the EC5M1 polypeptide of the
invention without having a nucleotide sequence as given in WO 99/06423. In a

similar manner, a polynucleotide sequence may encode the (full length) ECSM4 polypeptide of the invention without having the same sequence as that given in Figure 4 or Figure 5 or Figure 12. Such polynucleotides are within the scope of this invention.

5

Hence, it will be appreciated that a polynucleotide of the thirteenth aspect of the invention is preferably not one whose nucleotide sequence is given in Figure 4, and that a polynucleotide of the fourteenth aspect of the invention is preferably not a polynucleotide which is disclosed in WO 99/06423, such as
10 SEQ ID No 32 disclosed therein or its complement or variants or the corresponding cDNA sequence deposited under Accession No 209145 at the ATCC or a polynucleotide fragment capable of encoding a polypeptide whose amino acid sequence comprises the sequence given in SEQ ID No 120 of WO 99/06423.

15

A polynucleotide of the thirteenth or fourteenth aspects of the invention may encode a variant of the ECSM4 or ECSM1 polypeptide as described above. In addition, the insertions and/or deletions within the ECSM4 or ECSM1 polypeptide may lead to frameshift mutations which may encode truncated (or
20 elongated) polypeptide products, and insertions, deletions or other mutations may lead to the introduction of stop codons which encode truncate polypeptide products.

The polynucleotide of the invention may be DNA or RNA. It is preferred if it
25 is DNA.

The polynucleotide may or may not contain introns. It is preferred if it does not contain introns.

5 The polynucleotide may be single stranded or double stranded or a mixture of either.

The polynucleotide of the invention has at least 10 nucleotides, and preferably at least 15 nucleotides and more preferably at least 30 nucleotides. In a further preference, the polynucleotide is more than 50 nucleotides, more preferably at
10 least 100 nucleotides, and still more preferably the polynucleotide is at least 500 nucleotides. The polynucleotide may be more than 1kb, and may comprise more than 5kb.

The invention also includes a polynucleotide which is able to selectively
15 hybridise to a polynucleotide which encodes the ECSM4 or ECSM1 polypeptide or a fragment or variant or fusion thereof, or a fusion of said variant or fragment. Preferably, said polynucleotide is at least 10 nucleotides, more preferably at least 15 nucleotides and still more preferably at least 30 nucleotides in length. The said polynucleotide may be longer than 100
20 nucleotides and may be longer than 200 nucleotides, but preferably the said polynucleotide is not longer than 250 nucleotides. Such polynucleotides are useful in procedures as a detection tool to demonstrate the presence of the polynucleotide in a sample. Such a sample may be a sample of DNA, such as a bacterial colony, fixed on a membrane or filter.

25

Preferably, the polynucleotide which is capable of selectively hybridising as said is not any one of the nucleotide sequences represented by SEQ ID No

18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00 53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of WO 99/11293.

- 5 By "selectively hybridise" we mean that the polynucleotide hybridises under conditions of high stringency. DNA-DNA, DNA-RNA and RNA-RNA hybridisation may be performed in aqueous solution containing between 0.1X SSC and 6X SSC and at temperatures of between 55°C and 70°C. It is well known in the art that the higher the temperature or the lower the SSC
- 10 concentration the more stringent the hybridisation conditions. By "high stringency" we mean 2X SSC and 65°C. 1X SSC is 0.15M NaCl/0.015M sodium citrate. Polynucleotides which hybridise at high stringency are included within the scope of the claimed invention.
- 15 In another embodiment, the polynucleotide can be used as a primer in the polymerase chain reaction (PCR), and in this capacity a polynucleotide of between 15 and 30 nucleotides is preferred. A polynucleotide of between 20 and 100 nucleotides is preferred when the fragment is to be used as a mutagenic PCR primer. It is particularly preferred if the PCR primer (when
- 20 not being used to mutate a nucleic acid) contains about 15 to 30 contiguous nucleotides (ie perfect matches) from the nucleotide sequence given in Figure 4 or Figure 7 or Figure 12 or Figure 13 from the nucleotide sequence given in Figure 2. Clearly, if the PCR primers are used for mutagenesis, differences compared to the sequence will be present.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* 239, 487-491) are preferred. Suitable PCR primers may have the following properties:

- 5 It is well known that the sequence at the 5' end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3' ends, as this feature
10 may promote the formation of an artifactual product called "primer dimer". When the 3' ends of the two primers hybridize, they form a "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

- 15 Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension
20 temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

Optimum annealing temperatures may be determined empirically and may be
25 higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid

will be stabilised. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1nM range.

- When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative to detecting the product of DNA amplification using agarose gel electrophoresis and ethidium bromide staining of the DNA, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by a PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The probe may be labelled with a radionuclide such as ^{32}P , ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991) "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* **195**, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* **15**, 152-157.
- PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.
- Fluorophore-quencher pairs are particularly suited to quantitative measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

Oligonucleotide primers can be synthesised using methods well known in the art, for example using solid-phase phosphoramidite chemistry.

- 5 A polynucleotide or oligonucleotide primer of the invention may contain one or more modified bases or may contain a backbone which has been modified for stability purposes or for other reasons. By modified we included for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA and these are included in the
- 10 scope of the invention.

In a preferred embodiment, the polynucleotides of the invention are detectably labelled. Suitable detectable labels are described in detail above.

- 15 A fifteenth aspect of the invention provides an expression vector comprising a polynucleotide as described above. Typically, the polynucleotides are those which encode the polypeptides ECSM1 or ECSM4 or a fragment, variant or fusion thereof.

- 20 By "expression vector" we mean one which is capable, in an appropriate host, of expressing a polypeptide encoded by the polynucleotide.

- Such vectors may be useful in expressing the encoded polypeptide in a host cell for production of useful quantities of the polypeptide, or may be useful in
- 25 medicine. Expression vectors comprising a polynucleotide according to the thirteenth or fourteenth aspects of the invention which are suitable for use in gene therapy are within the scope of the invention. Administration of a gene

therapy vector capable of expressing the ECSM4 polypeptide may be useful in modulating or inhibiting angiogenesis, since this polypeptide is likely to be a repulsive guidance receptor. Similarly, gene therapy vectors capable of expressing fragments or mutants of ECSM4 on the cell surface, which
5 fragments or mutants are capable of binding the ECSM4 cognate ligand but are not able to convey the normal downstream signal (for example, because the necessary cytosolic portion of the polypeptide is deleted or mutated so as to not be functional or capable of binding normally interacting cellular proteins) may also be useful in modulating angiogenesis in an individual.

10

Hence, in a preferred embodiment, the vector is one which is suitable for use in gene therapy. Examples of suitable vectors and methods of their introduction into cells are given in more detail below. In particular, the gene therapy methods and vectors described in relation to the use of promoters of ECSM4
15 may also be used in relation to the use of ECSM4 coding sequences or antisense in gene therapy.

It will be appreciated that the polynucleotide comprised within the expression vector of this aspect of the invention may be one which encodes the
20 polypeptide ECSM4 or ECSM1 or a fragment or variant thereof, or the polynucleotide may be one which is capable of selectively hybridising to the ECSM4 or ECSM1 coding region. Polynucleotides which are capable of hybridising to the ECSM4 or ECSM1 coding region are useful as antisense polynucleotides which may decrease the expression level of ECSM4 or
25 ECSM1 within a target cell. The design of suitable and effective antisense polynucleotides based on a known coding sequence is known in the art of gene therapy.

Preferably, the expression vector of this aspect of the invention is one which does not contain a polynucleotide sequence represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or
5 WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293 or their complement. Also preferably, the said vector is one which does not contain a polynucleotide encoding a polypeptide whose amino acid sequence is represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No
10 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293.

Both the amount of therapeutic protein or therapeutic polynucleotide produced and the duration of production are important issues in gene therapy.
15 Consequently, the use of viral vectors capable of cellular gene integration (eg retroviral vectors) may be more beneficial than non-integrating alternatives (eg adenovirus derived vectors) when repeated therapy is undesirable for immunogenicity reasons.

20 By "therapeutic polynucleotide" or "therapeutic protein" we include ECSM4 and ECSM1 coding sequences, the polypeptide product encoded by said coding sequences, and ECSM4 antisense polynucleotides. The therapeutic effect of said polynucleotides or proteins may include pro-angiogenic or anti-angiogenic effects, depending on the precise therapeutic agent administered. For example,
25 an expression vector suitable for gene therapy which comprises a polynucleotide which is antisense to at least part of the ECSM4 coding region may have anti-angiogenic activity when expressed in a host cell or patient if it

suppresses expression of a molecule which is required for angiogenesis. If the polynucleotide comprised within the expression vector encodes a polypeptide which is required for inhibition of angiogenesis (for example, because said polypeptide has endothelial cell repulsive guidance activity), then expression of
5 the antisense may also be anti-angiogenic.

Conversely, if said the expression vector comprises a polynucleotide of the invention which polynucleotide suppresses expression of a molecule whose activity is required to decrease vascular growth (for example, because said
10 molecule is an endothelial cell repulsive guidance molecule) or encodes a polypeptide whose activity is required for angiogenesis, administration of the said vector may be pro-angiogenic.

Where the therapeutic gene is maintained extrachromosomally, the highest
15 level of expression is likely to be achieved using viral promoters, for example, the Rous sarcoma virus long terminal repeat (Ragot *et al* (1993) *Nature* 361, 647-650; Hyde *et al* (1993) *Nature* 362, 250-255) and the adenovirus major late promoter. The latter has been used successfully to drive the expression of a cystic fibrosis transmembrane conductance regulator (CFTR) gene in lung
20 epithelium (Rosenfeld *et al* (1992) *Cell* 68, 143-155). Since these promoters function in a broad range of tissues they may not be suitable to direct cell-type-specific expression unless the delivery method can be adapted to provide the specificity. However, somatic enhancer sequences could be used to give cell-type-specific expression in an extrachromosomal setting.

25

As described in more detail below, the ECSM4 regulatory/promoter region is an example of a regulatory region capable of conferring endothelial cell

selective expression, preferably selective to endothelial cells of neovasculature (ie, angiogenic endothelial cells) on an operatively linked coding region. As outlined above, such a coding region may encode an antisense polynucleotide.

- 5 Where withdrawal of the gene-vector construct is not possible, it may be necessary to add a suicide gene to the system to abort toxic reactions rapidly. The herpes simplex virus thymidine kinase gene, when transduced into cells, renders them sensitive to the drug ganciclovir, creating the option of killing the cells quickly.

10

- The use of ectotropic viruses, which are species specific, may provide a safer alternative to the use of amphotropic viruses as vectors in gene therapy. In this approach, a human homologue of the non-human, ectotropic viral receptor is modified in such a way so as to allow recognition by the virus. The modified
15 receptor is then delivered to cells by constructing a molecule, the front end of which is specified for the targeted cells and the tail part being the altered receptor. Following delivery of the receptor to its target, the genetically engineered ectotropic virus, carrying the therapeutic gene, can be injected and will only integrate into the targeted cells.

20

- Virus-derived gene transfer vectors can be adapted to recognise only specific cells so it may be possible to target to an endothelial cell, such as endothelial cells within a tumour. Similarly, it is possible to target expression of an therapeutic gene to the endothelial cell, using an endothelial cell-specific
25 promoter such as that for the ECSM4 or ECSM1 genes.

One of the ECSM genes or a part of the genes or a polynucleotide comprising an antisense to the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation, the gene will be expressed by the cell from the extrachromosomal location. Vectors for
5 introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation and viral transduction are known in the art, and the choice of method is within the competence of the ordinary skilled person.
10 Cells transformed with the wild-type novel gene can be used as model systems to study cancer remission and drug treatments which promote such remission.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to vectors, for example, via complementary cohesive termini.
15 For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

20 Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E.coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic
25 activities, and fill in recessed 3'-ends with their polymerising activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a larger molar excess of linker molecules in the presence of an enzyme that is able to catalyse the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA
5 ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

10

Synthetic linkers containing a variety of restriction endonuclease site are commercially available from a number of sources including International Biotechnologies Inc., New Haven, CN, USA.

15 A desirable way to modify the DNA encoding the polypeptide of the invention is to use PCR. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

20 In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

25

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the polypeptide of the

invention. Thus, the DNA encoding the polypeptide constituting the polypeptide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crawl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case of retroviral vectors, RNA) encoding the polypeptide constituting the polypeptide of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally,

not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed
5 cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the expression vector of the invention
10 are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example, *E.coli*
15 and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors typically include a prokaryotic replicon, such as the ColE1 *ori*, for
20 propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E.coli*, transformed therewith.

25

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter

sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

- 5 Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

- 10 A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

- 15 An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

- 20 Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (Ycps).

25

Other vectors and expression systems are well known in the art for use with a variety of host cells.

A sixteenth aspect of the invention provides a recombinant host cell comprising a polynucleotide or vector of the invention.

- 5 The polynucleotide of the invention includes polynucleotides encoding a compound of the third aspect of the invention (where both the moiety which selectively binds and the further moiety are polypeptides which are fused) or an ECSM4 or ECSM1 polypeptide of the invention or a fragment or fusion or variant thereof as defined above.

10

- The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E.coli* such as, for example, the *E.coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American
15 Type Culture Collection (ATCC) of Rockville, MD, USA (No. ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems,
20 La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CRL 1658 and 293 cells which are human embryonic kidney cells. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

- 25 Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells,

see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory*
5 *Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

10

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cells, bacterial cells, insect cells and vertebrate cells.

15 For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5 PEB using 6250V per cm at 25 μ FD.

20

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194, 182.

25 Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well-known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can

be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described
5 below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of
10 the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity.

Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

15

The host cell may be a host cell within an animal body. Thus, transgenic animals which express a polypeptide of the first or third aspects of the invention by virtue of the presence of the transgene are included. Preferably, the transgenic animal is a rodent such as a mouse. Transgenic animals can be
20 made using methods well known in the art.

Polynucleotides encoding the polypeptide ECSM4 may be useful in generating transgenic non-human mammals wherein the ECSM4 is mutated in some way. For example, the mouse ECSM4 genomic coding region may be mutated in a
25 mouse so as to produce an ECSM4 polypeptide which is incapable of binding its natural ligand, or incapable of correctly interacting with intracellular components. Such a mutated ECSM4 polypeptide may produce a disease in

the mouse which is very similar to a disease involving abnormal vascularisation in humans.

Hence, non-human mammals, especially rodents such as mice and rats, are
5 useful as models of diseases involving abnormal vascularisation.

Alternatively, mammals lacking the ECSM4 gene ("knock-outs") or lacking an ECSM4 genomic coding region which is capable of being transcribed or of expressing the ECSM4 polypeptide, may be useful in providing a means of
10 generating antibodies selective for the human ECSM4 polypeptide. Such mammals, especially mice, are likely to be particularly useful since the high level of homology between the human and mouse ECSM4 polypeptides may prevent human ECSM4 polypeptide from being antigenic in mice who do express the ECSM4 polypeptide.

15

A potentially more accurate animal model of diseases involving abnormal vascularisation may be made by addition to the genome of a transgenic animal as described above, or replacing the genomic ECSM4 of an animal with, the gene for human ECSM4 which has been mutated. Suitably, the human ECSM4
20 inserted will be under control of an endothelial selective promoter and regulatory region. Preferably, the promoter and regulatory regions are those of the host animal ECSM4 gene. An animal whose genome is modified in this way will express the dysfunctional human ECSM4, and therefore will be useful in testing the efficacy of drugs and antibodies in the diagnosis, prognosis and
25 treatment of diseases involving abnormal vascularisation in humans.

Such knockout or transgenic mammals are within the scope of the invention and antibodies generated using such mammals and compounds comprising them are also included within the scope of the invention.

- 5 A seventeenth aspect of the invention provides a method of producing a polypeptide of the invention, the method comprising expressing a polynucleotide as described above or culturing a host cell as described herein.

It will be appreciated that in order to produce the ECSM1 polypeptide, the host
10 cell may comprise a polynucleotide encoding a polypeptide whose amino acid sequence includes the sequence given in Figure 2, and that in order to produce the ECSM4 polypeptide the host cell may comprise a polynucleotide encoding the polypeptide whose amino acid sequence is given in Figure 4 or Figure 7 or Figure 12 and so on.

15 Preferably, the polynucleotide expressed does not consist of any one of the nucleotide sequences represented by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00/53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 and SEQ ID No 31 of WO 99/11293.

20 Also preferably, the polypeptide produced is not one with an amino acid sequence consisting of the sequence represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or
25 SEQ ID No 86 of WO 99/11293.

Methods of cultivating host cells and isolating recombinant proteins are well known in the art. It will be appreciated that, depending on the host cell, the ECSM1 or ECSM4 polypeptides produced may differ from that which can be isolated from nature. For example, certain host cells, such as yeast or bacterial
5 cells, either do not have, or have different, post-translational modification systems which may result in the production of forms of ECSM1 or ECSM4 which may be post-translationally modified in a different way to ECSM1 or ECSM4 isolated from nature. In order to obtain ECSM1 or ECSM4 which is post-translationally modified in a different way to human ECSM1 or ECSM4 it
10 is preferred if the host cell is a non-human host cell; more preferably it is not a mammalian cell.

It is preferred that the ECSM1 or ECSM4 polypeptide is produced in a eukaryotic system, such as an insect cell.

15 According to a less preferred embodiment, the ECSM1 or ECSM4 polypeptide can be produced *in vitro* using a commercially available *in vitro* translation system, such as rabbit reticulocyte lysate or wheatgerm lysate (available from Promega). Preferably, the translation system is rabbit reticulocyte lysate.
20 Conveniently, the translation system may be coupled to a transcription system, such as the TNT transcription-translation system (Promega). This system has the advantage of producing suitable mRNA transcript from an encoding DNA polynucleotide in the same reaction as the translation. Conveniently, where the expressed polypeptide comprises one or more transmembrane domains, the
25 translation system can be supplemented with a source of endoplasmic reticulum-derived membranes and folding chaperones, such as dog pancreatic microsomes, to allow synthesis of the polypeptide in a native conformation.

Preferably, the production method of this aspect of the invention comprises a further step of isolating the ECSM1 or ECSM4 produced from the host cell or from the *in vitro* translation mix. Preferably, the isolation employs an antibody
5 which selectively binds the expressed polypeptide of the invention.

It will be understood that the invention comprises the ECSM1 or ECSM4 polypeptides or the variants or fragments or fusions thereof, or a fusion of said variants or fragments obtainable by the methods herein disclosed, provided that
10 the ECSM4 polypeptide is not one which consists of the amino acid sequence given in Figure 4. Preferably, the polypeptide is not one which consists of an amino acid sequence represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of
15 WO 99/11293. Preferably, the ECSM1 polypeptide produced by the methods herein disclosed is not one which is encoded by SEQ ID No 32 of WO 99/06423 or encoded by the nucleic acid of ATCC deposit No.209145 made on July 17 1997 for the purposes of WO 99/06423.

20 An eighteenth aspect of the invention provides an antibody capable of selectively binding to either ECSM4 or ECSM1 as defined above.

Preferably, an antibody which selectively binds ECSM1 is not one which binds a polypeptide encoded by SEQ ID No 32 of WO 99/06423 or encoded by the
25 nucleic acid of ATCC deposit No 209145 made on July 17 1997 for the purposes of the international patent application PCT/US98/15949.

Preferably, an antibody which selectively binds ECSM1 is one which binds a polypeptide whose amino acid sequence comprises the sequence given in Figure 2 or a natural variant thereof but does not comprise the amino acid sequence encoded by ATCC deposit No 209145 made on July 17 1997.

5

Preferably, an antibody which selectively binds ECSM4 is one which binds a polypeptide whose amino acid sequence comprises the sequence given in any one of Figures 4, 5, 7, 12 or 13 or a natural variant thereof but does not bind the polypeptide represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 10 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293, or encoded by any one of the nucleotide sequences represented by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00/53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 and SEQ ID 15 No 31 of WO 99/11293.

By "selectively bind" we include antibodies which bind at least 10-fold more strongly to a polypeptide of the invention (such as ECSM4 or ECSM1) than to another polypeptide; preferably at least 50-fold more strongly and more 20 preferably at least 100-fold more strongly. Such antibodies may be made by methods well known in the art using the information concerning the differences in amino acid sequence of ECSM4 or ECSM1 and another polypeptide which is not a polypeptide of the invention.

25 Antibodies which selectively bind ECSM4 may also modulate the function of the ECSM4 polypeptide. Antibodies which mimic the effect of binding of the cognate ligand by stimulating or activating ECSM4, or which bind and thereby

prevent subsequent binding and activation or stimulation of ECSM4 by the cognate ligand, and such function-modulating antibodies are included in the scope of the invention. It will be appreciated that antibodies which modulate the function are useful as a tool in research, for example in studying the effects of ECSM4 stimulation or activation, or downstream processes triggered by such stimulation. Such antibodies are also useful in medicine, for example in modulating angiogenesis in an individual. Specifically, modulation of angiogenesis by administration of such an antibody may be useful in the treatment of a disease in an individual where modulation of angiogenesis would be beneficial, such as cancer.

The following peptides may be useful as immunogens in the generation of antibodies, such as rabbit polyclonal sera: LSQSPGAVPQALVAWRA, DSVLTPEEVALCLEL, TYGYISVPTA and KGGVLLCPRPCLTPT.

In a preferred embodiment of this aspect, the antibody of the invention selectively binds an amino acid sequence with the sequence GGDSLLGGRGSL, LLQPPARGHAHDGQALSTD, EPQDYTEPVE, TAPGGQGAPWAE or ERATQEPSEHGP. These sequences represent amino acid sequences which are not identical between the human and mouse ECSM4 polypeptide sequences. Generally, the human and mouse ECSM4 polypeptides display a high degree of identity, which makes the production of mouse antibodies to the human ECSM4 particularly difficult due to the lack of immunogenicity of much of the human ECSM4 sequence in mouse. Amino acid sequences which are absent from the mouse ECSM4 are more likely to more be immunogenic in a mouse than those sequences which are present in the mouse ECSM4 (an alignment of the human and mouse ECSM4 amino acid

sequences is shown in Figure 14). Hence, polypeptide fragments which contain sequences which are unique to human ECSM4 as described above are more useful than ECSM4 polypeptides whose sequence is found in both human and mouse ECSM4, in the production of antibodies which selectively bind the
5 human ECSM4 polypeptide.

Antibodies generated as a result of use of amino acid sequences which are located in the extracellular portion of the ECSM4 polypeptide are likely to be useful as endothelial cell targeting molecules. Therefore, it is particularly
10 preferred if the antibody of the invention is raised to, and preferably selectively binds, an amino acid sequence which is unique to the human ECSM4 polypeptide, which sequence is located towards the N-terminal end of the polypeptide and is found in the extracellular portion located between residues 1 and 467 of the amino acid sequence given in Figure 12. An example of an
15 amino acid sequence which is suitable for raising antibody molecules selective for the ECSM4 extracellular region is given in Figure 12.

Although the amino acid sequences which are unique to the human ECSM4 may be used to produce polyclonal antibodies, it is preferred if they are used to
20 produce monoclonal antibodies.

Peptides in which one or more of the amino acid residues are chemically modified, before or after the peptide is synthesised, may be used providing that the function of the peptide, namely the production of specific antibodies *in*
25 *vivo*, remains substantially unchanged. Such modifications included forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amid of a terminal carboxyl group,

and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from in vivo metabolism. The peptides may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to
5 obviate the use of a carrier. It may be advantageous for the peptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the peptide is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that
10 the peptide of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody
15 (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-
20 Thr-Asn-Cys, β -galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different peptides of the invention may be cross-linked to one another; in this situation there is no
25 separate carrier as such, but a carrier function may be provided by such cross-linking. Suitably cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and

succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present).

If the peptide is prepared by expression of a suitable nucleotide sequence in a
5 suitable host, then it may be advantageous to express the peptide as a fusion product with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase
10 peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* 46, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl
15 ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-
20 dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked
25 agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added

using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

The peptide of the invention may be linked to other antigens to provide a dual effect.

Included in the scope of the invention is a method of producing an antibody according to this aspect of the invention.

Antibodies can be raised in an animal by immunising with an appropriate peptide. Appropriate peptides are described herein. Alternatively, with today's technology, it is possible to make antibodies as defined herein without the need to use animals. Such techniques include, for example, antibody phage display technology as is well known in the art. Appropriate peptides, as described herein, may be used to select antibodies produced in this way.

It will be appreciated that, with the advancements in antibody technology, it may not be necessary to immunise an animal in order to produce an antibody. Synthetic systems, such as phage display libraries, may be used. The use of such systems is included in the methods of the invention and the products of such systems are "antibodies" for the purposes of the invention.

It will be appreciated that such antibodies which recognise ECSM1 or ECSM4 and variants or fragments thereof are useful research reagents and therapeutic agents, particularly when prepared as a compound of the invention as described above. Suitably, the antibodies of the invention are detectably labelled, for example they may be labelled in such a way that they may be directly or indirectly detected. Conveniently, the antibodies are labelled with a radioactive moiety or a coloured moiety or a fluorescent moiety, or they may be linked to an enzyme. Typically, the enzyme is one which can convert a non-coloured (or non-fluorescent) substrate to a coloured (or fluorescent) product. The antibody may be labelled by biotin (or streptavidin) and then detected indirectly using streptavidin (or biotin) which has been labelled with a radioactive moiety or a coloured moiety or a fluorescent moiety, or the like or they may be linked to any enzyme of the type described above.

A nineteenth aspect of the invention provides a method of detecting endothelial damage or activation in an individual comprising obtaining a fluid sample from the individual and detecting the presence of fragments of ECSM1 or ECSM4 in the sample.

5

Preferably, the fluid sample is blood. Typically, the presence of peptide fragments derived from ECSM1 or ECSM4 are detected.

In a preferred embodiment of this aspect, the presence of peptide fragments of the ECSM1 or ECSM4 polypeptides are detected using an antibody selective for a polypeptide whose amino acid sequence comprises a sequence given in either one of Figure 2 or Figure 4 or Figure 12 or fragments thereof. Preferably, the antibody is an antibody according to the eighteenth aspect of the invention. Typically, such an antibody would be detectably labelled.

15

Detecting or diagnosing endothelial cell damage in an individual is useful in diagnosing cancer or aiding diagnosis of cardiac disease, endometriosis or arteriosclerosis in that individual. It may be that certain levels of apparent cell damage are detected in individuals who do not have cancer, cardiac disease, endometriosis or arteriosclerosis. It may be necessary to compare the amount of endothelial cell damage detected with amounts or levels observed in individuals who are known to have cancer, cardiac disease, endometriosis or arteriosclerosis with the "normal" levels of apparent damage in the individual who does not have cancer, cardiac disease, endometriosis or arteriosclerosis.

20

Hence, detection of endothelial damage or activation in an individual may be useful as a means of detecting the presence or extent or growth rate of a tumour

in that individual. The detection of vessel damage is an indirect report of the formation of tumour neovasculature. In this way, ECSM4 or ECSM1 may be surrogate markers of angiogenesis. The presence of ECSM4 or ECSM1 fragments in a sample from the individual, or more ECSM4 or ECSM1 polypeptide fragments than in an individual who does not have a tumour, may be a means of detecting a tumour, or growth of a known tumour, in that individual.

Furthermore, it will be appreciated that detection of neovasculature by means of detecting the presence of, or a certain level of, ECSM4 or ECSM1 in a sample from an individual may be useful in determining if a treatment in that individual is being effective, and/or to what extent the treatment is effective. Preferably the therapy is to treat a tumour or cancer in the individual.

Hence, an aspect of the invention provides a method of detecting a tumour or tumour neovasculature or cardiac disease or endometriosis or arteriosclerosis in an individual comprising obtaining a fluid sample from the individual and detecting the presence of fragments of ECSM1 or ECSM4 in the sample.

As described above in relation to detecting or diagnosing endothelial cell damage, detection of the disease (such as a tumour or cardiac disease etc) by means of detecting the presence of, or a certain level of, ECSM4 or ECSM1 in a sample from an individual may be useful in determining the efficacy of a treatment in that individual.

In one embodiment, the therapy is gene therapy.

Preferably, the efficacy of the a treatment in an individual is determined using the amount of fragments of ECSM1 or ECSM4 found in the fluid sample of the individual and comparing it to either to the amount of ECSM1 or ECSM4 fragments in a sample from an individual who does not have cancer, cardiac
5 disease, endometriosis or arteriosclerosis and/or to the amount in a sample from the individual prior to commencement of said treatment. The comparison indicates the efficacy of treatment of the individual, wherein if there is no change in the amount of fragments determined before and during/after treatment this is indicative of poor efficacy of the treatment. A decrease in the
10 amount of fragments found during or after treatment compared to the amount found before treatment was started indicates some efficacy of the treatment in ameliorating the condition of the individual.

Current methods of assessing the efficacy of various anti-angiogenic therapies
15 being tested in clinical trials are invasive. The selective expression of ECSM4 on endothelial cells of angiogenic blood vessels means that detecting the presence, absence, increase or decrease in the level of ECSM1 or ECSM4 in a subject undergoing therapy is a means of determining the efficacy of the therapy in that subject without the need, or with a reduced need, for invasive
20 biopsies, scans and the such like.

Hence, determination of the level of ECSM1 and or ECSM4 fragments in the blood of an individual undergoing an anti-angiogenic therapy (such as cancer therapy) may act as a "surrogate marker of angiogenesis".

25

By "peptide fragments derived from ECSM1 or ECSM4" we mean peptides which have at least 5 consecutive amino acids of the ECSM4 or ECSM1

polypeptide. Typically, the fragments have at least 8 consecutive amino acids, preferably at least 10, more preferably at least 12 or 15 or 20 or 30 or 40 or 50 consecutive amino acids of the ECSM4 or ECSM1 polypeptide.

- 5 Methods for detecting the presence of fragments of peptides derived from larger polypeptides are known in the art.

A further aspect of the invention provides a method of modulating angiogenesis in an individual, the method comprising administering to the
10 individual ECSM4 or a peptide fragment of ECSM4 or a ligand of ECSM4 or an antibody which selectively binds to ECSM4 or ECSM1.

Preferably, the peptide fragment or ligand or antibody is one which modulates the activity or function, either directly or indirectly, of the ECSM4 polypeptide
15 of the individual.

Preferred antibodies are those as described in more detail above.

The production of antibodies which modulate the function of a polypeptide exposed on the cell surface is known in the art and is discussed in more detail
20 above. Such antibodies may modulate the function by imitating the function of the natural ligand and stimulating the polypeptide into activity or function, or may modulate the polypeptide function by preventing stimulation of the polypeptide by the ligand by sterically obscuring the ligand binding site
25 thereby preventing binding of the natural ligand.

Delivery of a ligand to magic roundabout might be an angiogenic inhibitor useful in therapy of cancer or other diseases involving hyper-angiogenesis. Also, introduction of the ECSM4 polypeptide to endothelial cells by gene therapy using the ECSM4 encoding polynucleotide might alter growth and
5 migration.

A still further aspect of the invention provides a method of diagnosing a condition which involves aberrant or excessive growth of vascular endothelium in an individual comprising obtaining a sample containing nucleic acid from
10 the individual and contacting said sample with a polynucleotide which selectively hybridises to a nucleic acid which encodes the ECSM4 polypeptide or the ECSM1 polypeptide or a fragment or natural variant thereof.

The method may be used for aiding diagnosis.

15

A condition which involves aberrant or excessive growth of vascular endothelium such as cancer, arteriosclerosis, restenosis, diabetic retinopathy, arthritis, psoriasis, endometriosis, menorrhagia, haemangiomas and venous malformations may be caused by a mutation in the nucleic acid which encodes
20 the ECSM1 or ECSM4 polypeptides.

By "selectively hybridising" is meant that the nucleic acid has sufficient nucleotide sequence similarity with the said human DNA or cDNA that it can hybridise under moderately or highly stringent conditions. As is well known in
25 the art, the stringency of nucleic acid hybridization depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridizing sequences and on factors such as temperature, ionic strength and

CG or AT content of the sequence. Thus, any nucleic acid which is capable of selectively hybridising as said is useful in the practice of the invention.

Nucleic acids which can selectively hybridise to the said human DNA or
5 cDNA include nucleic acids which have >95% sequence identity, preferably those with >98%, more preferably those with >99% sequence identity, over at least a portion of the nucleic acid with the said human DNA or cDNA. As is well known, human genes usually contain introns such that, for example, a mRNA or cDNA derived from a gene within the said human DNA would not
10 match perfectly along its entire length with the said human DNA but would nevertheless be a nucleic acid capable of selectively hybridising to the said human DNA. Thus, the invention specifically includes nucleic acids which selectively hybridise to an ECSM4 or ECSM1 cDNA but may not hybridise to an ECSM4 or ECSM1 gene, or *vice versa*. For example, nucleic acids which
15 span the intron-exon boundaries of the ECSM4 or ECSM1 gene may not be able to selectively hybridise to the ECSM4 or ECSM1 cDNA.

Typical moderately or highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in
20 *Molecular Cloning, a laboratory manual*, 2nd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is
25 immobilised on a nylon membrane and the probe nucleic acid is ≥ 500 bases or base pairs is:

6 x SSC (saline sodium citrate)

0.5% sodium dodecyl sulphate (SDS)

100 µg/ml denatured, fragmented salmon sperm DNA

- 5 The hybridisation is performed at 68°C. The nylon membrane, with the nucleic acid immobilised, may be washed at 68°C in 1 x SSC or, for high stringency, 0.1 x SSC.

- 20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and
10 88.2 g of sodium citrate in 800 ml of H₂O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H₂O. Dispense into aliquots. Sterilize by autoclaving.

- An example of a typical hybridisation solution when a nucleic acid is
15 immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M sodium phosphate (pH 6.8)

- 20 1 mm EDTA (pH 7.6)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

0.1% nonfat dried milk

- 25 The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al*

(1988) *Nucl. Acids Res.* 16, 4637 discusses the determination of T_m s. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

- 5 By "nucleic acid which selectively hybridises" is also included nucleic acids which will amplify DNA from the said region of human DNA by any of the well known amplification systems such as those described in more detail below, in particular the polymerase chain reaction (PCR). Suitable conditions for PCR amplification include amplification in a suitable 1 x amplification
10 buffer:

10 x amplification buffer is 500 mM KCl; 100 mM Tris.Cl (pH 8.3 at room temperature); 15 mM $MgCl_2$; 0.1% gelatin.

- 15 A suitable denaturing agent or procedure (such as heating to 95°C) is used in order to separate the strands of double-stranded DNA.

Suitably, the annealing part of the amplification is between 37°C and 60°C, preferably 50°C.

20

- Although the nucleic acid which is useful in the methods of the invention may be RNA or DNA, DNA is preferred. Although the nucleic acid which is useful in the methods of the invention may be double-stranded or single-stranded, single-stranded nucleic acid is preferred under some circumstances such as in
25 nucleic acid amplification reactions.

The sample may be directly derived from the patient, for example, by biopsy of a tissue which may be associated with aberrant vascular development, or it may be derived from the patient from a site remote from the tissue, for example because cells from the tissue have migrated from the tissue to other parts of the
5 body. Alternatively, the sample may be indirectly derived from the patient in the sense that, for example, the tissue or cells therefrom may be cultivated *in vitro*, or cultivated in a xenograft model; or the nucleic acid sample may be one which has been replicated (whether *in vitro* or *in vivo*) from nucleic acid from the original source from the patient. Thus, although the nucleic acid derived
10 from the patient may have been physically within the patient, it may alternatively have been copied from nucleic acid which was physically within the patient. When aberrant vascular development is believed to be associated with a tumour, tumour tissue may be taken from the primary tumour or from metastases.

15

It will be appreciated that a useful method of the invention includes the analysis of mutations in, or the detection of the presence or absence of, the ECSM4 or ECSM1 gene in any suitable sample. The sample may suitably be a freshly-obtained sample from the patient, or the sample may be an historic
20 sample, for example a sample held in a library of samples.

Conveniently, the nucleic acid capable of selectively hybridising to the said human DNA and which is used in the methods of the invention further comprises a detectable label.

25

By "detectable label" is included any convenient radioactive label such as ^{32}P , ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using

well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be
5 detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless *o*-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed
10 assay and whether the nucleic acid hybridises to the said region of human DNA can be determined by reference to the position of hybridisation in the fixed assay. The detectable label may also be a fluorophore-quencher pair as described in Tyagi & Kramer (1996) *Nature Biotechnology* 14, 303-308.

15 Conveniently, in this method of diagnosis of a condition in which vascular development is aberrant the nucleic acid which is capable of the said selective hybridisation (whether labelled with a detectable label or not) is contacted with a nucleic acid derived from the patient under hybridising conditions. Suitable hybridising conditions include those described above.

20

This method of diagnosing a condition in which vascular development is aberrant may involve sequencing of DNA at one or more of the relevant positions within the relevant region, including direct sequencing; direct sequencing of PCR-amplified exons; differential hybridisation of an
25 oligonucleotide probe designed to hybridise at the relevant positions within the relevant region (conveniently this uses immobilised oligonucleotide probes in, so-called, "chip" systems which are well known in the art); denaturing gel

- electrophoresis following digestion with an appropriate restriction enzyme, preferably following amplification of the relevant DNA regions; S1 nuclease sequence analysis; non-denaturing gel electrophoresis, preferably following amplification of the relevant DNA regions; conventional RFLP (restriction
5 fragment length polymorphism) assays; heteroduplex analysis; selective DNA amplification using oligonucleotides; fluorescent *in-situ* hybridisation (FISH) of interphase chromosomes; ARMS-PCR (Amplification Refractory Mutation System-PCR) for specific mutations; cleavage at mismatch sites in hybridised nucleic acids (the cleavage being chemical or enzymic); SSCP single strand
10 conformational polymorphism or DGGE (discontinuous or denaturing gradient gel electrophoresis); analysis to detect mismatch in annealed normal/mutant PCR-amplified DNA; and protein truncation assay (translation and transcription of exons - if a mutation introduces a stop codon a truncated protein product will result). Other methods may be employed such as detecting
15 changes in the secondary structure of single-stranded DNA resulting from changes in the primary sequence, for example, using the cleavase I enzyme. This system is commercially available from GibcoBRL, Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, Scotland.
- 20 It will be appreciated that the methods of the invention may also be carried out on "DNA chips". Such "chips" are described in US 5,445,934 (Affymetrix; probe arrays), WO 96/31622 (Oxford; probe array plus ligase or polymerase extension), and WO 95/22058 (Affymax; fluorescently marked targets bind to oligomer substrate, and location in array detected); all of these are incorporated
25 herein by reference.

Detailed methods of mutation detection are described in "Laboratory Protocols for Mutation Detection" 1996, ed. Landegren, Oxford University Press on behalf of HUGO (Human Genome Organisation).

- 5 It is preferred if RFLP is used for the detection of fairly large (≥ 500 bp) deletions or insertions. Southern blots may be used for this method of the invention.

10 PCR amplification of smaller regions (maximum 300bp) to detect small changes greater than 3-4 bp insertions or deletions may be preferred. Amplified sequence may be analysed on a sequencing gel, and small changes (minimum size 3-4 bp) can be visualised. Suitable primers are designed as herein described.

- 15 In addition, using either Southern blot analysis or PCR restriction enzyme variant sites may be detected. For example, for analysing variant sites in genomic DNA restriction enzyme digestion, gel electrophoresis, Southern blotting, and hybridisation specific probe (for example any suitable fragment derived from the ECSM4 or ECSM1 cDNA or gene).

20

For example, for analysing variant sites using PCR DNA amplification, restriction enzyme digestion, gel detection by ethidium bromide, silver staining or incorporation of radionucleotide or fluorescent primer in the PCR.

- 25 Other suitable methods include the development of allele specific oligonucleotides (ASOs) for specific mutational events. Similar methods are used on RNA and cDNA for the suitable tissue.

Whilst it is useful to detect mutations in any part of the ECSM4 or ECSM1 gene, it is preferred if the mutations are detected in the exons of the gene and it is further preferred if the mutations are ones which change the coding sense.

5 The detection of these mutations is a preferred aspect of the invention.

The methods of the invention also include checking for loss-of-heterozygosity (LOH; shows one copy lost). LOH may be a sufficient marker for diagnosis; looking for mutation/loss of the second allele may not be necessary. LOH of

10 the gene may be detected using polymorphisms in the coding sequence, and introns, of the gene.

Particularly preferred nucleic acids for use in the aforementioned methods of the invention are those selected from the group consisting of primers suitable

15 for amplifying nucleic acid.

Suitably, the primers are selected from the group consisting of primers which hybridise to the nucleotide sequences shown in any of the Figures which show ECSM4 or ECSM1 gene or cDNA sequences. It is particularly preferred if the

20 primers hybridise to the introns of the ECSM4 or ECSM1 gene or if the primers are ones which will prime synthesis of DNA from the ECSM4 or ECSM1 gene or cDNA but not from other genes or cDNAs.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki

25 *et al* (1988) *Science* 239, 487-491) are preferred. Suitable PCR primers and methods of detecting products of PCR reactions are described in detail above.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton (1991) *Nature* 350, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* 20, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

10 The present invention provides the use of a nucleic acid which selectively hybridises to the human-derived DNA of genomic clones as described in Table 8 of Example 1 or to the ECSM4 or ECSM1 gene, or a mutant allele thereof, or a nucleic acid which selectively hybridises to ECSM4 or ECSM1 cDNA or a mutant allele thereof, or their complement in a method of diagnosing a
15 condition in which vascular development is aberrant; or in the manufacture of a reagent for carrying out these methods.

Preferred polynucleotides which selectively hybridise to the ECSM4 gene or cDNA are as described above in relation to a method of diagnosis.

20

Also, the present invention provides a method of determining the presence or absence, or mutation in, the said ECSM4 or ECSM1 gene. Preferably, the method uses a suitable sample from a patient.

25 The methods of the invention include the detection of mutations in the ECSM4 or ECSM1 gene.

The methods of the invention may make use of a difference in restriction enzyme cleavage sites caused by mutation. A non-denaturing gel may be used to detect differing lengths of fragments resulting from digestion with an appropriate restriction enzyme.

5

An "appropriate restriction enzyme" is one which will recognise and cut the wild-type sequence and not the mutated sequence or *vice versa*. The sequence which is recognised and cut by the restriction enzyme (or not, as the case may be) can be present as a consequence of the mutation or it can be introduced into
10 the normal or mutant allele using mismatched oligonucleotides in the PCR reaction. It is convenient if the enzyme cuts DNA only infrequently, in other words if it recognises a sequence which occurs only rarely.

In another method, a pair of PCR primers are used which match (ie hybridise
15 to) either the wild-type genotype or the mutant genotype but not both. Whether amplified DNA is produced will then indicate the wild-type or mutant genotype (and hence phenotype). However, this method relies partly on a negative result (ie the absence of amplified DNA) which could be due to a technical failure. It therefore may be less reliable and/or requires additional
20 control experiments.

A preferable method employs similar PCR primers but, as well as hybridising to only one of the wild-type or mutant sequences, they introduce a restriction site which is not otherwise there in either the wild-type or mutant sequences.

25

The nucleic acids which selectively hybridise to the ECSM4 or ECSM1 gene or cDNA, or which selectively hybridise to the genomic clones containing

ECSM4 or ECSM1 as listed in Table 8 of Example 1 are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in the RNase protection method for detecting point mutations already discussed above. The probes can be used to detect PCR amplification products. They
5 may also be used to detect mismatches with the ECSM4 or ECSM1 gene or mRNA in a sample using other techniques. Mismatches can be detected using either enzymes (eg S1 nuclease or resolvase), chemicals (eg hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids. These techniques
10 are known in the art. Generally, the probes are complementary to the ECSM4 or ECSM1 gene coding sequences, although probes to certain introns are also contemplated. A battery of nucleic acid probes may be used to compose a kit for detecting loss of or mutation in the wild-type ECSM4 or ECSM1 gene. The kit allows for hybridization to the entire ECSM4 or ECSM1 gene. The
15 probes may overlap with each other or be contiguous.

If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the human ECSM4 or ECSM1 gene. The riboprobe thus is an anti-sense probe in that it does not code for the protein encoded by the ECSM4
20 or ECSM1 gene because it is of the opposite polarity to the sense strand. The riboprobe generally will be labelled, for example, radioactively labelled which can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches.

25

Nucleic acid probes may also be complementary to mutant alleles of the ECSM4 or ECSM1 gene. These are useful to detect similar mutations in other

patients on the basis of hybridization rather than mismatches. As mentioned above, the ECSM4 or ECSM1 gene probes can also be used in Southern hybridizations to genomic DNA to detect gross chromosomal changes such as deletions and insertions.

5

Particularly useful methods of detecting a mutation in the ECSM1 or ECSM4 genes include single strand conformation polymorphism (SSCP), hetero duplex analysis, polymerase chain reaction, using DNA chips and sequencing.

10 Any sample containing nucleic acid derived from the individual is useful in the methods of the invention. It is preferred if the nucleic acid in the sample is DNA. Thus, samples from cells may be obtained as is well known in the art, for example from blood samples or cheek cells or the like. Where the methods are being used to determine the presence or absence of a mutation in an unborn
15 child, it is preferred if the sample is a maternal sample containing nucleic acid from the unborn child. Suitable maternal samples include the amniotic fluid of the mother, chorionic villus samples and blood samples from which foetal cells can be isolated.

20 A further aspect of the invention provides a method of reducing the expression of the ECSM4 or ECSM1 polynucleotide in an individual, comprising administering to the individual an agent which selectively prevents expression of ECSM4 or ECSM1.

25 In a preferred embodiment, the agent which selectively prevents expression of ECSM4 or ECSM1 is an antisense nucleic acid.

Preferably, the antisense nucleic acid is not one (or is not antisense to one) whose sequence consists of the sequence represented by SEQ ID No 18084 or 5096 of EP 1 074 617, SEQ ID No 210 of WO 00/53756 or WO 99/46281, or SEQ ID Nos 22, 23, 96 or 98 of WO 01/23523 or SEQ ID No 31 of
5 WO 99/11293 or their complement, or a nucleic acid sequence which encodes a polypeptide whose amino acid sequence is represented by any one of SEQ ID No 18085 of EP 1 074 617, SEQ ID No 211 of either WO 00/53756 or WO99/46281, SEQ ID Nos 24-27, 29, 30, 33, 34, 38 or 39 of WO 01/23523, or SEQ ID No 86 of WO 99/11293.

10 A further aspect thereof includes administering an antisense nucleic acid to a cell in order to prevent expression of ECSM4 or ECSM1. Typically, the cell is in the body of an individual in need of prevention of expression of ESCM4 or ECSM1.

15 The ECSM4 or ECSM1 polynucleotide which is bound by an antisense molecule may be DNA or RNA.

Preferred antisense molecules are as described above.

20 Diseases which may be treated by reducing ECSM4 or ECSM1 expression include diseases involving aberrant or excessive vascularisation as described above.

25 Antisense nucleic acids are well known in the art and are typically single-stranded nucleic acids, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a

DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that
5 oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise a sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

- 10 By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A)addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

15

- Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after transfection with plasmids or retroviruses or other vectors carrying an antisense gene. Antisense
20 oligonucleotides were first discovered to inhibit viral replication or expression in cell culture for Rous sarcoma virus, vesicular stomatitis virus, herpes simplex virus type 1, simian virus and influenza virus. Since then, inhibition of mRNA translation by antisense oligonucleotides has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ
25 extracts. Inhibition of viral function by antisense oligonucleotides has been demonstrated *in vitro* using oligonucleotides which were complementary to the AIDS HIV retrovirus RNA (Goodchild, J. 1988 "Inhibition of Human

Immunodeficiency Virus Replication by Antisense Oligodeoxynucleotides", *Proc. Natl. Acad. Sci. (USA)* 85(15), 5507-11). The Goodchild study showed that oligonucleotides that were most effective were complementary to the poly(A) signal; also effective were those targeted at the 5' end of the RNA, particularly the cap and 5' untranslated region, next to the primer binding site and at the primer binding site. The cap, 5' untranslated region, and poly(A) signal lie within the sequence repeated at the ends of retrovirus RNA (R region) and the oligonucleotides complementary to these may bind twice to the RNA.

10

Typically, antisense oligonucleotides are 15 to 35 bases in length. For example, 20-mer oligonucleotides have been shown to inhibit the expression of the epidermal growth factor receptor mRNA (Witters *et al*, *Breast Cancer Res Treat* 53:41-50 (1999)) and 25-mer oligonucleotides have been shown to decrease the expression of adrenocorticotrophic hormone by greater than 90% (Frankel *et al*, *J Neurosurg* 91:261-7 (1999)). However, it is appreciated that it may be desirable to use oligonucleotides with lengths outside this range, for example 10, 11, 12, 13, or 14 bases, or 36, 37, 38, 39 or 40 bases.

20 Oligonucleotides are subject to being degraded or inactivated by cellular endogenous nucleases. To counter this problem, it is possible to use modified oligonucleotides, eg having altered internucleotide linkages, in which the naturally occurring phosphodiester linkages have been replaced with another linkage. For example, Agrawal *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 7079-7083 showed increased inhibition in tissue culture of HIV-1 using oligonucleotide phosphoramidates and phosphorothioates. Sarin *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 7448-7451 demonstrated increased inhibition of

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HIV-1 using oligonucleotide methylphosphonates. Agrawal *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7790-7794 showed inhibition of HIV-1 replication in both early-infected and chronically infected cell cultures, using nucleotide sequence-specific oligonucleotide phosphorothioates. Leither *et al* (1990) *Proc.*
5 *Natl. Acad. Sci. USA* **87**, 3430-3434 report inhibition in tissue culture of influenza virus replication by oligonucleotide phosphorothioates.

Oligonucleotides having artificial linkages have been shown to be resistant to degradation *in vivo*. For example, Shaw *et al* (1991) in *Nucleic Acids Res.* **19**,
10 747-750, report that otherwise unmodified oligonucleotides become more resistant to nucleases *in vivo* when they are blocked at the 3' end by certain capping structures and that uncapped oligonucleotide phosphorothioates are not degraded *in vivo*.

15 A detailed description of the H-phosphonate approach to synthesizing oligonucleoside phosphorothioates is provided in Agrawal and Tang (1990) *Tetrahedron Letters* **31**, 7541-7544, the teachings of which are hereby incorporated herein by reference. Syntheses of oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters,
20 bridged phosphoramidates and bridge phosphorothioates are known in the art. See, for example, Agrawal and Goodchild (1987) *Tetrahedron Letters* **28**, 3539; Nielsen *et al* (1988) *Tetrahedron Letters* **29**, 2911; Jager *et al* (1988) *Biochemistry* **27**, 7237; Uznanski *et al* (1987) *Tetrahedron Letters* **28**, 3401; Bannwarth (1988) *Helv. Chim. Acta.* **71**, 1517; Crosstick and Vyle (1989)
25 *Tetrahedron Letters* **30**, 4693; Agrawal *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1401-1405, the teachings of which are incorporated herein by reference. Other methods for synthesis or production also are possible. In a preferred

embodiment the oligonucleotide is a deoxyribonucleic acid (DNA), although ribonucleic acid (RNA) sequences may also be synthesized and applied.

The oligonucleotides useful in the invention preferably are designed to resist
5 degradation by endogenous nucleolytic enzymes. *In vivo* degradation of oligonucleotides produces oligonucleotide breakdown products of reduced length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to use oligonucleotides that are resistant to
10 degradation in the body and which are able to reach the targeted cells. The present oligonucleotides can be rendered more resistant to degradation *in vivo* by substituting one or more internal artificial internucleotide linkages for the native phosphodiester linkages, for example, by replacing phosphate with sulphur in the linkage. Examples of linkages that may be used include phosphorothioates,
15 methylphosphonates, sulphone, sulphate, ketyl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are illustrative, rather than limiting, since other internucleotide linkages are known in the art. See, for example, Cohen, (1990) *Trends in Biotechnology*. The synthesis of oligonucleotides having one or
20 more of these linkages substituted for the phosphodiester internucleotide linkages is well known in the art, including synthetic pathways for producing oligonucleotides having mixed internucleotide linkages.

Oligonucleotides can be made resistant to extension by endogenous enzymes by
25 "capping" or incorporating similar groups on the 5' or 3' terminal nucleotides. A reagent for capping is commercially available as Amino-Link IITM from Applied BioSystems Inc, Foster City, CA. Methods for capping are described, for

example, by Shaw *et al* (1991) *Nucleic Acids Res.* **19**, 747-750 and Agrawal *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**(17), 7595-7599, the teachings of which are hereby incorporated herein by reference.

- 5 A further method of making oligonucleotides resistant to nuclease attack is for them to be "self-stabilized" as described by Tang *et al* (1993) *Nucl. Acids Res.* **21**, 2729-2735 incorporated herein by reference. Self-stabilized oligonucleotides have hairpin loop structures at their 3' ends, and show increased resistance to degradation by snake venom phosphodiesterase, DNA polymerase I and fetal
- 10 bovine serum. The self-stabilized region of the oligonucleotide does not interfere in hybridization with complementary nucleic acids, and pharmacokinetic and stability studies in mice have shown increased *in vivo* persistence of self-stabilized oligonucleotides with respect to their linear counterparts.
- 15 In accordance with the invention, the antisense compound may be administered systemically. Alternatively the inherent binding specificity of antisense oligonucleotides characteristic of base pairing is enhanced by limiting the availability of the antisense compound to its intended locus *in vivo*, permitting lower dosages to be used and minimising systemic effects. Thus,
- 20 oligonucleotides may be applied locally to achieve the desired effect. The concentration of the oligonucleotides at the desired locus is much higher than if the oligonucleotides were administered systemically, and the therapeutic effect can be achieved using a significantly lower total amount. The local high concentration of oligonucleotides enhances penetration of the targeted cells and
- 25 effectively blocks translation of the target nucleic acid sequences.

The oligonucleotides can be delivered to the locus by any means appropriate for localised administration of a drug. For example, a solution of the oligonucleotides can be injected directly to the site or can be delivered by infusion using an infusion pump. The oligonucleotides also can be incorporated
5 into an implantable device which when placed at the desired site, permits the oligonucleotides to be released into the surrounding locus.

The oligonucleotides may be administered via a hydrogel material. The hydrogel is non-inflammatory and biodegradable. Many such materials now are known,
10 including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular
15 weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10% to about 80% by weight ethylene oxide and from about 20% to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be
20 used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^R.

In this embodiment, the hydrogel is cooled to a liquid state and the oligonucleotides are admixed into the liquid to a concentration of about 1 mg
25 oligonucleotide per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms,

it solidifies to form a gel, and the oligonucleotides diffuse out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

- 5 It will be appreciated that the oligonucleotides or other agents may be administered after surgical removal of a tumour, and may be administered to the area from which the tumour has been removed, and surrounding tissue, for example using cytосcopy to guide application of the oligonucleotides or other agents.

10

- The oligonucleotides can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the oligonucleotides. The oligonucleotides can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.
- 15
20

- The dose of oligonucleotides is dependent on the size of the oligonucleotides and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of oligonucleotide is somewhat dependent on the length and chemical composition of the
- 25

oligonucleotide but is generally in the range of about 30 to 3000 μg per square centimetre of tissue surface area.

The oligonucleotides may be administered to the patient systemically for both
5 therapeutic and prophylactic purposes. The oligonucleotides may be administered by any effective method, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the oligonucleotides to access and circulate in the patient's bloodstream. Oligonucleotides administered systemically preferably are given in
10 addition to locally administered oligonucleotides, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

15 It will be appreciated that antisense agents also include larger molecules which bind to said ECSM4 or ECSM1 mRNA or genes and substantially prevent expression of said ECSM4 or ECSM1 mRNA or genes and substantially prevent expression of said ECSM4 or ECSM1 protein. Thus, expression of an antisense molecule which is substantially complementary to said ECSM4 or ECSM1
20 mRNA is envisaged as part of the invention.

The said larger molecules may be expressed from any suitable genetic construct as is described below and delivered to the patient. Typically, the genetic construct which expresses the antisense molecule comprises at least a portion of
25 the said ECSM4 or ECSM1 cDNA or gene operatively linked to a promoter which can express the antisense molecule in a cell. Promoters that may be active in endothelial cells are described below.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

Preferably, the genetic construct is adapted for delivery to a human cell.

5

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into proliferating endothelial cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the endothelial cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* 16, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting proliferating endothelial cells because they can only integrate into the genome of dividing cells; most endothelial cells are in a quiescent, non-receptive stage of cell growth or, at least, are dividing much less rapidly than angiogenic cells. Retroviral DNA constructs which encode said antisense agents may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo*^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 µm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 µg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is

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appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml.

Alternatively, as described in Culver *et al* (1992) *Science* **256**, 1550-1552, cells
5 which produce retroviruses are injected into specific tissue. The retrovirus-
producing cells so introduced are engineered to actively produce retroviral
vector particles so that continuous productions of the vector occurred within
the tumour mass *in situ*. Thus, proliferating endothelial cells can be
successfully transduced *in vivo* if mixed with retroviral vector-producing cells.

10

Targeted retroviruses are also available for use in the invention; for example,
sequences conferring specific binding affinities may be engineered into pre-
existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a
review of this and other targeted vectors for gene therapy).

15

Other methods involve simple delivery of the construct into the cell for
expression therein either for a limited time or, following integration into the
genome, for a longer time. An example of the latter approach includes
(preferably endothelial-cell-targeted) liposomes (Nässander *et al* (1992)
20 *Cancer Res.* **52**, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in
targeting to endothelial cell types which express a cell surface protein for
which antibodies are available.

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Other methods of delivery include adenoviruses carrying external DNA via an
antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and

transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type
5 adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the cell and carries into the cell with it the DNA construct of the invention. It is
10 preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

15 In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently
20 linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic
25 constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of

the invention are supplied to the endothelial cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other
5 genetic constructs of the invention using the endosome-disruption activity of
defective or chemically inactivated adenovirus particles produced by the
methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* 89, 6094-6098 may
also be used. This approach appears to rely on the fact that adenoviruses are
adapted to allow release of their DNA from an endosome without passage
10 through the lysosome, and in the presence of, for example transferrin linked to
the DNA construct or other genetic construct of the invention, the construct is
taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex
15 retroviral constructs; there is no permanent modification of the genome as
occurs with retroviral infection; and the targeted expression system is coupled
with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse a tumour with the suitable delivery
20 vehicle comprising the genetic construct for a period of time; additionally or
alternatively the delivery vehicle or genetic construct can be injected directly
into accessible tumours.

It will be appreciated that "naked DNA" and DNA complexed with cationic
25 and neutral lipids may also be useful in introducing the DNA of the invention
into cells of the patient to be treated. Non-viral approaches to gene therapy are
described in Ledley (1995) *Human Gene Therapy* 6, 1129-1144.

Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al*
5 (1995) *Gene Therapy* 2, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* 274, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a
10 further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

In a further embodiment the agent which selectively prevents the function of
15 ECSM4 or ECSM1 is a ribozyme capable of cleaving targeted ECSM4 or ECSM1 RNA or DNA. A gene expressing said ribozyme may be administered in substantially the same and using substantially the same vehicles as for the antisense molecules.

20 Ribozymes which may be encoded in the genomes of the viruses or virus-like particles herein disclosed are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNase P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" US 5,149,796; Cech *et al* "RNA ribozyme restriction
25 endoribonucleases and methods", US 5,116,742; Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endonucleases and methods", US 5,093,246; and Been *et al* "RNA ribozyme polymerases, dephosphorylases,

restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference.

- 5 It will be appreciated that it may be desirable that the antisense molecule or ribozyme is expressed from a cell-specific promoter element.

The genetic constructs of the invention can be prepared using methods well known in the art.

10

A further aspect of the invention is a method of screening for a molecule that binds to ECSM4 or a suitable variant, fragment or fusion thereof, or a fusion of a said fragment or fusion thereof, the method comprising 1) contacting a) the ECSM4 polypeptide with b) a test molecule 2) detecting the presence of a
15 complex containing the ECSM4 polypeptide and a test molecule, and optionally 3) identifying any test molecule bound to the ECSM4 polypeptide.

Preferably the ECSM4 polypeptide is one as described above in respect of the eleventh aspect of the invention.

20

In a preferred embodiment, the test molecule is a polypeptide.

In a further preferred embodiment, the method is used to identify natural ligands of ECSM4. Thus, in this embodiment the test molecule includes the
25 natural ligand of ECSM4. A particularly useful technique for the identification of natural ligands of polypeptide molecules is the yeast two-hybrid technique. This technique is well known in the art and relies on binding between a

molecule and its cognate ligand to bring together two parts of a transcription complex (which are fused one to the molecule in question and other to the test ligand) which, when together, promote transcription of a reporter gene.

- 5 Hence, a preferred embodiment of this aspect of the invention comprises use of the screening method, preferably the yeast two-hybrid system, to identify natural ligands of the ECSM4 polypeptide.

A molecule which is identifiable as binding the ECSM4 polypeptide is a
10 further aspect of the invention.

It will be appreciated that a molecule which binds to ECSM4 may modulate the activation of ECSM4.

- 15 Suitable peptide ligands that will bind to ECSM4 may be identified using methods known in the art.

One method, disclosed by Scott and Smith (1990) *Science* 249, 386-390 and Cwirla *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 6378-6382, involves the
20 screening of a vast library of filamentous bacteriophages, such as M13 or fd, each member of the library having a different peptide fused to a protein on the surface of the bacteriophage. Those members of the library that bind to ECSM4 are selected using an iterative binding protocol, and once the phages that bind most tightly have been purified, the sequence of the peptide ligands may be determined
25 simply by sequencing the DNA encoding the surface protein fusion. Another method that can be used is the NovaTope (TM) system commercially available from Novagen, Inc., 597 Science Drive, Madison, WI 53711. The method is

based on the creation of a library of bacterial clones, each of which stably expresses a small peptide derived from a candidate protein in which the ligand is believed to reside. The library is screened by standard lift methods using the antibody or other binding agent as a probe. Positive clones can be analysed
5 directly by DNA sequencing to determine the precise amino acid sequence of the ligand.

Further methods using libraries of beads conjugated to individual species of peptides as disclosed by Lam *et al* (1991) *Nature* 354, 82-84 or synthetic peptide
10 combinatorial libraries as disclosed by Houghten *et al* (1991) *Nature* 354, 84-86 or matrices of individual synthetic peptide sequences on a solid support as disclosed by Pirrung *et al* in US 5143854 may also be used to identify peptide ligands.

15 It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system may be used. For example, an assay for identifying a compound capable of modulating the activity of a
20 protein kinase may be performed as follows. Beads comprising scintillant and a polypeptide that may be phosphorylated may be prepared. The beads may be mixed with a sample comprising the protein kinase and ^{32}P -ATP or ^{33}P -ATP and with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known
25 parameters for ^{32}P or ^{33}P SPA assays. Only ^{32}P or ^{33}P that is in proximity to the scintillant, i.e. only that bound to the polypeptide, is detected. Variants of such

an assay, for example in which the polypeptide is immobilised on the scintillant beads *via* binding to an antibody, may also be used.

Other methods of detecting polypeptide/polypeptide interactions include
5 ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring the interaction of the fluorescent labels when in close proximity to
10 each other.

Alternative methods of detecting binding of a polypeptide to macromolecules, for example DNA, RNA, proteins and phospholipids, include a surface plasmon resonance assay, for example as described in Plant *et al* (1995) *Analyt*
15 *Biochem* 226(2), 342-348. Methods may make use of a polypeptide that is labelled, for example with a radioactive or fluorescent label.

A further method of identifying a compound that is capable of binding to the ECSM4 polypeptide is one where the polypeptide is exposed to the compound
20 and any binding of the compound to the said polypeptide is detected and/or measured. The binding constant for the binding of the compound to the polypeptide may be determined. Suitable methods for detecting and/or measuring (quantifying) the binding of a compound to a polypeptide are well known to those skilled in the art and may be performed, for example, using a
25 method capable of high throughput operation, for example a chip-based method. New technology, called VLSIPS™, has enabled the production of extremely small chips that contain hundreds of thousands or more of different

molecular probes. These biological chips or arrays have probes arranged in arrays, each probe assigned a specific location. Biological chips have been produced in which each location has a scale of, for example, ten microns. The chips can be used to determine whether target molecules interact with any of the probes on the chip. After exposing the array to target molecules under selected test conditions, scanning devices can examine each location in the array and determine whether a target molecule has interacted with the probe at that location.

10 Biological chips or arrays are useful in a variety of screening techniques for obtaining information about either the probes or the target molecules. For example, a library of peptides can be used as probes to screen for drugs. The peptides can be exposed to a receptor, and those probes that bind to the receptor can be identified. See US Patent No. 5,874,219 issued 23 February 15 1999 to Rava *et al.*

Another method of targeting proteins that modulate the activity of ECSM4 is the yeast two-hybrid system, where the polypeptides of the invention can be used to "capture" ECSM4 protein binding proteins. The yeast two-hybrid 20 system is described in Fields & Song, *Nature* 340:245-246 (1989).

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the 25 interactions between the said and the interacting polypeptide are substantially the same as between a said naturally occurring polypeptide and a naturally occurring interacting polypeptide *in vivo*.

It will be appreciated that in the method described herein, the ligand may be a drug-like compound or lead compound for the development of a drug-like compound.

5

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that
10 may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able
15 to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for
20 use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

25 Alternatively, the methods may be used as "library screening" methods, a term well known to those skilled in the art. Thus, for example, the method of the invention may be used to detect (and optionally identify) a polynucleotide

capable of expressing a polypeptide activator of ECSM4. Aliquots of an expression library in a suitable vector may be tested for the ability to give the required result.

- 5 Hence, an embodiment of this aspect of the invention provides a method of identifying a drug-like compound or lead compound for the development of a drug-like compound that modulates the activity of the polypeptide ECSM4, the method comprising contacting a compound with the polypeptide or a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment
10 or derivative thereof and determining whether, for example, the enzymic activity of the said polypeptide is changed compared to the activity of the said polypeptide or said variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof in the absence of said compound.
- 15 Preferably, the ECSM4 polypeptide is as described above in respect of the eleventh aspect of the invention.

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood
20 that reagents and conditions used in the method may be chosen such that the interactions between the said polypeptide and its substrate are substantially the same as *in vivo*.

In one embodiment, the compound decreases the activity of said polypeptide.
25 For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of said polypeptide. In a further example, the compound may bind to a portion of said polypeptide that is not the active site

so as to interfere with the binding of the said polypeptide to its ligand. In a still further example, the compound may bind to a portion of said polypeptide so as to decrease said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural
5 regulation of the said polypeptide's activity, for example in the activation of the said polypeptide by an "upstream activator".

A still further aspect of the invention provides a polynucleotide comprising a promoter and/or regulatory portion of any one of the ECSM1 or ECSM4 genes.

10

By "ECSM1 or ECSM4 genes" we mean the natural genomic sequence which when transcribed is capable of encoding a polypeptide comprising the ECSM1 or ECSM4 polypeptide sequence as defined herein. The natural genomic sequence of the ECSM1 or ECSM4 genes may contain introns.

15

The polynucleotide of this aspect of the invention is preferably one which has transcriptional promoter activity. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Preferably the transcriptional promoter activity is
20 present in mammalian cells and more preferably the polynucleotide has transcriptional promoter activity in endothelial cells. In a preferred embodiment, the transcriptional promoter activity is present in endothelial cells and not in other cell types.

25 Preferably, the promoter and/or regulatory portion is one which can direct endothelial cell selective expression.

Preferably, the promoter or regulatory region of the ECSM4 gene is one which is capable of promoting transcription of an operatively-linked coding sequence in response to hypoxic conditions. More preferably, the level of transcription of the coding sequence is up-regulated in hypoxic conditions compared to the level of transcription in the absence of hypoxia. By "hypoxic conditions" we include the physiological conditions of cancer where the inappropriate cell proliferation deprives surrounding tissue of oxygen, cardiac disease where for example a vessel occlusion may restrict the delivery of oxygen to certain tissues, and tissue necrosis where destruction of vascular tissue cells results in a reduced supply of oxygen to surrounding tissue and the consequent death of that surrounding tissue. Hypoxia is described in more detail in Hockel and Vaupel (2001) *J. Nat. Can. Inst.* 93: 266-276.

Hence, in a preferred embodiment, the ECSM4 promoter or regulatory region is comprised in a vector suitable for use in gene therapy for driving expression of a therapeutic gene to treat a hypoxic condition. Preferably, the hypoxic condition is cancer or cardiac disease. A "therapeutic gene" may be any gene which provides a desired therapeutic effect.

It will be appreciated that use of the said ECSM4 promoter to treat a hypoxic condition, for example by gene therapy, is included within the scope of the present invention.

Methods for the determination of the sequence of the promoter region of a gene are well known in the art. The presence of a promoter region may be determined by identification of known motifs, and confirmed by mutational analysis of the identified sequence. Preferably, the promoter sequence is

located in the region 5kb upstream of the genomic coding region of ECSM1 or ECSM4. More preferably, it is located in the region 3kb or 2 kb or 1 kb or 500bp upstream, and still more preferably it is located within 210 bp of the transcription start site.

5

Regulatory regions, or transcriptional elements such as enhancers are less predictable than promoters in their location relative to a gene. However, many motifs indicative of regulatory regions are well characterised and such regions affecting the level of transcription of the relevant gene can usually be identified
10 on the basis of these motifs. The function of such a region can be demonstrated by well-known methods such as mutational analysis and in vitro DNA-binding assays including DNA footprinting and gel mobility shift assays.

Regulatory regions influencing the transcription of the ECSM1 or ECSM4
15 genes are likely to be located within the region 20 kb or 10 kb or 7 kb 5 kb or 3 kb, or more preferably 1 kb 5' upstream of the relevant genomic coding region or can be located within introns of the gene.

Sequence tagged sites and mapping intervals will be helpful in localising
20 promoter regions, regulatory regions and physical clones.

In a further preferred embodiment, the polynucleotide comprising the promoter and/or regulatory portion is operatively linked to a polynucleotide encoding a polypeptide. Methods for linking promoter polynucleotides to polypeptide
25 coding sequences are well known in the art.

Preferably the polypeptide is a therapeutic polypeptide. A therapeutic polypeptide may be any polypeptide which it is medically useful to express selectively in endothelial cells. Examples of such therapeutic polypeptides include anti-proliferative, immunomodulatory or blood clotting-influencing factors, or anti-proliferative or anti-inflammatory cytokines. They may also comprise anti-cancer polypeptides.

In one embodiment of this aspect of the invention, the polynucleotide is one suitable for use in medicine. Thus, the invention includes the polynucleotide packaged and presented for use in medicine. It will be appreciated that such polynucleotides will be especially useful in gene therapy, especially where it is desirable to express a therapeutic polypeptide selectively an endothelial cell. It is preferred if the polynucleotide is one suitable for use in gene therapy.

Gene therapy may be carried out according to generally accepted methods, for example, as described by Friedman, 1991. A virus or plasmid vector (see further details below), containing a copy of the gene to be expressed linked to expression control elements such as promoters and other regulatory elements influencing transcription of ECSM1 or ECSM4 as described above and capable of replicating inside endothelial cells, is prepared. Suitable vectors are known, such as disclosed in US Patent 5,252,479 and WO 93/07282. The vector is then injected into the patient, either locally or systemically. If the transfected gene is not permanently incorporated into the genome of each of the targeted endothelial cells, the treatment may have to be repeated periodically.

25

Gene transfer systems known in the art may be useful in the practice of the gene therapy methods of the present invention. These include viral and

- nonviral transfer methods. A number of viruses have been used as gene transfer vectors, including papovaviruses, eg SV40 (Madzak *et al*, 1992), adenovirus (Berkner, 1992; Berkner *et al*, 1988; Gorziglia and Kapikian, 1992; Quantin *et al*, 1992; Rosenfeld *et al*, 1992; Wilkinson *et al*, 1992; Stratford-
5 Perricaudet *et al*, 1990), vaccinia virus (Moss, 1992), adeno-associated virus (Muzyczka, 1992; Ohi *et al*, 1990), herpesviruses including HSV and EBV (Margolskee, 1992; Johnson *et al*, 1992; Fink *et al*, 1992; Breakfield and Geller, 1987; Freese *et al*, 1990), and retroviruses of avian (Brandyopadhyay and Temin, 1984; Petropoulos *et al.*, 1992), murine (Miller, 1992; Miller *et al*,
10 1985; Sorge *et al*, 1984; Mann and Baltimore, 1985; Miller *et al*, 1988), and human origin (Shimada *et al*, 1991; Helseth *et al*, 1990; Page *et al*, 1990; Buchschacher and Panganiban, 1992). To date most human gene therapy protocols have been based on disabled murine retroviruses.
- 15 Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Graham and van der Eb, 1973; Pellicer *et al*, 1980); mechanical techniques, for example microinjection (Anderson *et al*, 1980; Gordon *et al*, 1980; Brinster *et al*, 1981; Constantini and Lacy, 1981); membrane fusion-mediated transfer *via* liposomes (Felgner *et al*,
20 1987; Wang and Huang, 1989; Kaneda *et al*, 1989; Stewart *et al*, 1992; Nabel *et al*, 1990; Lim *et al*, 1992); and direct DNA uptake and receptor-mediated DNA transfer (Wolff *et al*, 1990; Wu *et al*, 1991; Zenke *et al*, 1990; Wu *et al*, 1989b; Wolff *et al*, 1991; Wagner *et al*, 1990; Wagner *et al*, 1991; Cotten *et al*, 1990; Curiel *et al*, 1991a; Curiel *et al*, 1991b).

Other suitable systems include the retroviral-adenoviral hybrid system described by Feng *et al* (1997) *Nature Biotechnology* 15, 866-870, or viral systems with targeting ligands such as suitable single chain Fv fragments.

- 5 In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and
10 degradation of the endosome before the coupled DNA is damaged.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is nonspecific, localized *in vivo* uptake and expression have
15 been reported in tumour deposits, for example, following direct *in situ* administration (Nabel, 1992).

Gene transfer techniques which target DNA directly to tissues, eg endothelial cells, is preferred. Receptor-mediated gene transfer, for example, is
20 accomplished by the conjugation of DNA (usually in the form of covalently closed supercoiled plasmid) to a protein ligand *via* polylysine. Ligands are chosen on the basis of the presence of the corresponding ligand receptors on the cell surface of the target cell/tissue type. In the case of endothelial cells, a suitable receptor is ECSM4. These ligand-DNA conjugates can be injected
25 directly into the blood if desired and are directed to the target tissue where receptor binding and internalization of the DNA-protein complex occurs. To

overcome the problem of intracellular destruction of DNA, coinfection with adenovirus can be included to disrupt endosome function.

- In the case where replacement gene therapy using a functionally wild-type gene is used, it may be useful to monitor the treatment by detecting the presence of replacement gene mRNA or encoded replacement polypeptide, or functional gene product, at various sites in the body, including the endothelial cells, blood serum, and bodily secretions/excretions, for example urine.
- 10 A further aspect of the present invention provides a method of treating an individual with cancer, cardiac disease, a hypoxic condition, endometriosis or arteriosclerosis comprising administering to the individual a polynucleotide according to the invention, which polynucleotide comprises a promoter or regulatory region of the invention operatively linked to a polynucleotide
15 encoding a therapeutic polypeptide.

- A still further aspect of the invention provides a method of modulating angiogenesis in an individual comprising administering to the individual a polynucleotide according to the invention, which polynucleotide comprises a
20 promoter or regulatory region of the invention operatively linked to a polynucleotide encoding a therapeutic polypeptide or a polynucleotide which is capable of expressing ECSM4 or a fragment or variant thereof or which comprises an ECSM4 antisense nucleic acid.

- 25 The therapeutic polypeptide may be any therapeutic polypeptide which is useful in treating the individual. Preferably, the therapeutic polypeptide is any

one or more of immunomodulatory, anti-cancer, a blood-clotting-influencing factor or an anti-proliferative or anti-inflammatory cytokine.

Antisense nucleic acid is discussed in more detail above. Briefly, the function
5 of an antisense nucleic acid is to inhibit the translation of a specific mRNA to which the antisense nucleic acid is complementary and able to hybridise to within a cell, at least in part. The design of optimal antisense nucleic acid molecules is well known in the art of molecular biology.

10 The present invention also provides a use of a polynucleotide according to the invention, which polynucleotide comprises a promoter or regulatory region of the invention operatively linked to a polynucleotide encoding a therapeutic polypeptide in the manufacture of a medicament for treating cancer, cardiac disease, a hypoxic condition, endometriosis or arteriosclerosis.

15

The invention will now be described in more detail by reference to the following Examples and Figures herein

Figure 1.

20 Experimental verification by reverse transcription PCR. Candidate endothelial specific genes predicted by the combination of the UniGene/EST screen and xProfler SAGE differential analysis (Table 8) were checked for expression in three endothelial and nine non-endothelial cell cultures. Endothelial cultures were as follows: HMVEC (human microvascular endothelial cells), HUVEC
25 (human umbilical vein endothelial cells) confluent culture and HUVEC proliferating culture. Non-endothelial cultures were as follows: normal endometrial stromal (NES) cells grown in normoxia and NES grown in

hypoxia, MDA 453 and MDA 468 breast carcinoma cell lines, HeLa, FEK4 fibroblasts cultured in normoxia and FEK4 fibroblasts cultured in hypoxia, and SW480, HCT116 - two colorectal epithelium cell lines. ECSM1 showed complete endothelial specificity, while magic roundabout/ECSM4 was very strongly preferentially expressed in the endothelium. Interestingly, both these novel genes appear more endothelial specific than the benchmark endothelial specific gene: von Willebrand factor.

Figure 2.

- Phrap generated contig sequence for ECSM1 and amino acid sequence of the translation product. The ESTs used to generate this contig are shown in Table 10.

Figure 3.

- ECSM4 *in vitro* transcription/translation. The cDNA coding for full length ECSM4 was cloned into pBluescript plasmid vector. Circular and HindIII digested plasmid were subjected to *in vitro* transcription/translation using TNT[®] T7 Quick Coupled Transcription/Translation System (Promega Corporation) incorporating ³⁵S Methionine as per manufacturer's instructions. The reaction products were resolved by SDS PAGE and visualised by autoradiography. The Luciferase plasmid was utilised as a positive control for the reaction. The numbers on the left indicate the position of molecular size markers for reference. The size of the band denoting ECSM4 is consistent with the calculated molecular weight of the polypeptide of 118 kDa.

Figure 4.

cDNA and computer translation of GenBank AK000805 (human ECSM4/magic roundabout).

5 Figure 5.

Phrap generated contig sequence for human ECSM4 (magic roundabout) ESTs and translation of the encoded polypeptide. The DNA sequence is shown in the orientation as if it were a cDNA, which is opposite to that in which it was originally generated. The ESTs used to generate the contig are shown in Table
10 11. Translation start in this sequence is at position 2 of the contig sequence, and translation finish is at position 948.

Figure 6.

An alignment of the GenBank Accession No AK000805 ("magic.seq") and
15 Phrap ("hs.111518") generated nucleic acid sequences of human ECSM4 given in Figure 4 and 5.

Figure 7.

Mouse ECSM4 contig nucleotide sequence and amino acid sequence.
20

Figure 8.

An alignment of the amino acid sequences of the mouse Robo1 protein ("T30805") and human ECSM4 ("magic.pep").

25 Figure 9.

An alignment of the amino acid sequences of mouse Robo1 protein ("T30805") and mouse ECSM4 ("mousemagic.pep").

Figure 10.

An alignment of the amino acid sequences of human ("magic.pep") and mouse ("mousemagic.pep") ECSM4 proteins. Residues in bold indicate well
 5 conserved sequences. The mouse protein sequence is shown on top and the human sequence is below.

Figure 11.

Expression of magic roundabout *in vitro*. (a) Ribonuclease protection
 10 analysis. Top, two probes to different regions (nucleotides 1 to 355 and 3333 to 3679) of magic roundabout were used in the analysis (shown left and right). RNase protection assay was performed with U6 small nuclear RNA as control (shown bottom) (Maxwell *et al* (1999) *Nature* 399: 271). Human
 15 cell lines and primary isolates: MRC-5, fibroblast cell line, MCF-7, breast carcinoma cell line, Neuro, SY-SH-5Y neuroblastoma cell line, HUVEC, umbilical vein endothelial isolate, HDMEC, dermal microvascular endothelial isolate and HMME2, mammary microvascular endothelial cell line. N, normoxia, H, hypoxia, P, proliferating. (b) Western analysis of
 20 cell lysates. A band at ~ 110 kD corresponds to MR and was stronger in cells exposed to hypoxia for 18 h. The experiment was repeated twice with similar results. Immunoblotting was carried out as described in Brown *et al* (2000) *Cancer Res.* 60: 6298. Polyclonal rabbit anti-sera was raised against the following peptides coupled to keyhole limpet haemocyanin: amino acids
 165-181 (LSQSPGAVPQALVAWRA) and 274-288
 25 (DSVLTPPEEVALCLEL) (anti-sera 1) or peptides 311-320 (TYGYISVPTA) and 336-351 (KGGVLLCPPRPCLTPT) (anti-sera 2). Both anti-sera gave identical results. For western analysis, anti-sera was affinity purified on a

"Hi-Trap NHS-activated HP" column (Amersham) to which the peptides used to raise anti-sera 1 were coupled.

Figure 12.

- 5 Human ECSM4 full-length cDNA and encoded protein sequence.

Figure 13.

Mouse ECSM4 full-length cDNA (MuMR.seq) and encoded protein sequence.

- 10 Figure 14.

Alignment of human ECSM4 (top) and mouse ECSM4 (bottom) amino acid sequences.

Figure 15.

- 15 Alignment of human ECSM4 ("HuMR.seq"; top) and mouse ECSM4 ("MuMR.seq"; bottom) cDNA sequences.

Figure 16.

In situ hybridisation analysis of human placental tissue using ECSM4 as probe.

- 20 A bright field view of 10x magnification of thin section of placental tissue. The arrow indicates a large blood vessel.

Figure 17.

In situ hybridisation analysis of human placental tissue using ECSM4 as probe.

- 25 A higher magnification of the bright-field view of thin section of placental tissue shown in Figure 16, focussing on the blood vessel. The arrow points to endothelial cells lining the lumen of the vessel.

Figure 18.

In situ hybridisation analysis of human placental tissue using ECSM4 as probe.

A higher magnification of the thin section of placental tissue shown in Figure
5 16, focussing on the blood vessel and shown here in dark-field. The arrow
depicts positive staining of endothelial cells lining the lumen of the vessel.

Figure 19.

In situ hybridisation analysis of colorectal liver metastatic tissue using ECSM4

10 as probe. A bright-field view of a section of colorectal liver metastatic tissue
magnified with (A) 10x and (B) 20x objective. The area marked by the
boundary (encircling * A) depicts the normal liver tissue. The arrow in (B)
shows one of the blood vessels within the metastatic tumour tissue.

15 Figure 20.

In situ hybridisation analysis of colorectal liver metastatic tissue using ECSM4
as a probe. This is a dark field view of a section of colorectal liver metastatic
tissue magnified with (A) 10x and (B) 20x objective. The area marked by the
boundary (encircling *) depicts the normal liver tissue. The arrow in (B)
20 shows one of the blood vessels within the metastatic tumour tissue
corresponding to the vessel shown in Figure 19B. Expression of ECSM4 is
restricted to endothelial cells of the tumour blood vessels. Note that there is
little expression in the surrounding normal tissue (*).

Figure 21.

Western Blot using the rabbit antibody MGO-5 as primary antibody. Dilutions of the peptides ECSM4-derived peptides MR 165, MR 311, MR 366 and the control polypeptide Bovine Serum Albumin (BSA) were resolved by SDS polyacrylamide gel electrophoresis and blotted onto Immobilon P membrane. The blot was probed with MGO-5 antibody and visualised using anti-rabbit antibody coupled with alkaline phosphatase.

Figure 22.

Immunostaining of frozen placental section. A frozen thin section of human placenta was analysed by immunohistochemistry without any primary antibody (negative control) and visualised using anti-rabbit antibody coupled with alkaline phosphatase. Little background staining is observed.

Figure 23.

Immunostaining of frozen placental section. A frozen thin section of human placenta was analysed by immunohistochemistry using a primary antibody recognising von Willibrand Factor (positive control), and visualised using an anti-rabbit secondary antibody coupled with alkaline phosphatase. The arrows show high levels of expression of vWF restricted to the vascular endothelial cells.

Figure 24.

Immunostaining of frozen placental section. A frozen thin section of human placenta was analysed by immunohistochemistry using MGO-5 (a rabbit polyclonal antibody raised against peptide MR 165) as the primary antibody, and visualised using anti-rabbit secondary antibody coupled with alkaline

phosphatase. The arrows show high levels of expression of ECSM4 restricted to the vascular endothelial cells. Note that the surrounding tissue shows little staining. Comparison with Figure 22 and 23 shows that the expression of ECSM4 colocalises with that of vWF, a known marker for vascular endothelial cells.

Figure 25.

Immunohistochemical analysis of HUVEC cells: von Willibrand Factor (vWF). HUVEC cells were immobilised and analysed by immunohistochemistry using an antibody recognising von Willibrand Factor (a marker for endothelial cells) as the primary antibody and visualised using anti-rabbit antibody coupled with alkaline phosphatase. The arrows show expression of vWF in a subset of the HUVEC cells.

Figure 26.

Immunohistochemical analysis of HUVEC cells using the antibody MGO-7. HUVEC cells were immobilised and analysed by immunohistochemistry using MGO-7 antibody (a rabbit polyclonal antibody raised against peptides MR 311 and MR 336) as the primary antibody and visualised using anti-rabbit antibody coupled with alkaline phosphatase. The arrows show expression of ECSM4 in a subset of the HUVEC cells. Note that the staining is localised primarily to the cell surface of the cells.

Figure 27. Expression of magic roundabout *in vivo*.

(A) Expression of MR detected by *in situ* hybridisation in of a placental arteriole (a) and venule (b) (left, light field and right, dark field). (c) Immunohistochemical staining of magic roundabout in a placental arteriole.

Left, von Willibrand factor control and right, magic roundabout. (B) Expression of MR in tumour endothelium. Ganglioglioma (a) x20 and (b) x50. Left, light field; right, dark field. Arrows highlight a vessel running diagonally down the section with an erythrocyte within it. Endothelial cells are strongly positive for MR expression. Papillary bladder carcinoma (c) x20 and (d) x50. The vascular core of the papilla of the tumour is strongly positive, particularly the 'flat' endothelial cells indicated by arrows. A magic roundabout antisense *in situ* probe was generated using T3 polymerase from IMAGE EST clone 1912098 (GenBank acc. AI278949). The plasmid was linearised with Eco RI prior to probe synthesis. *In situ* analysis was then performed as described in Poulson *et al* (1998) *Eur. J. Histochemistry* 42:121-132.

Example 1.

15 *In silico* cloning of novel endothelial specific genes.

We describe the use of two independent strategies for differential expression analysis combined with experimental verification to identify genes specifically or preferentially expressed in vascular endothelium.

20

The first strategy was based the EST cluster expression analysis in the human UniGene gene index (Schuler *et al*, 1997). Recurrent gapped BLAST searches (Altschul *et al*, 1997) were performed at very high stringency against expressed sequence tags (ESTs) grouped in two pools. These two pools comprised endothelial cell and non-endothelial cell libraries derived from dbEST
25 (Boguski *et al*, 1995). The second strategy employed a second datamining tool: SAGEmap xProfiler. XProfiler is a freely available on-line tool, which is

a part of the NCBI's Cancer Genome Anatomy Project (CGAP) (Strausberg *et al*, 1997, Cole *et al*, 1995). While these two approaches alone were producing a discouragingly high number of false positives, when both strategies were combined, predictions proved exceptionally reliable and two novel candidate
5 endothelial-specific genes have been identified. Full-length cDNAs have been identified in sequence databases. Another gene (EST cluster) corresponds to a partial cDNA sequence from a large-scale cDNA sequencing project and contains a region of similarity to the intracellular domain of human roundabout homologue 1 (ROBO1).

10

UniGene/EST gene index screen

A pool of endothelial and a pool of non-endothelial sequences were extracted using Sequence Retrieval System (SRS) version 5 from dbEST. The endothelial pool consisted of 11,117 ESTs from nine human endothelial
15 libraries (Table 1). The non-endothelial pool included 173,137 ESTs from 108 human cell lines and microdissected tumour libraries (Table 2). ESTs were extracted from dbEST release April 2000. Multiple FASTA files were transformed into a BLAST searchable database using the pressdb programme. Table 3 shows the expression status of five known endothelial cell-specific
20 genes in these two pools.

Subsequently, the longest, representative sequence in each UniGene cluster (UniGene Build #111 May 2000, multiple FASTA file hs.seq.uniq) was searched using very high stringency BLAST against these two pools. If such
25 representative sequence reported no hits, the rest of the sequences belonging to the cluster (UniGene multiple-FASTA file hs.seq) were used as BLAST

queries. Finally, clusters with no hits in the non-endothelial pool and at least one hit in the endothelial pool were selected.

Optimising the BLAST E-value was crucial for the success of BLAST identity-level searches. Too high an E-value would result in gene paralogues being reported. On the other hand, too low (stringent) an E-parameter would result in many false negatives, i.e. true positives would not be reported due to sequencing errors in EST data: ESTs are large-scale low-cost single pass sequences and have high error rate (Aaronson *et al*, 1996). In this work an E-value of 10e-20 was used in searches against non-endothelial EST pool and a more stringent 10e-30 value in searches against the smaller endothelial pool. These values were deemed optimal after a series of test BLAST searches.

SAGE data and SAGEmap xProfiler differential analysis

Web-based SAGE library subtraction (SAGEmap xProfiler: <http://www.ncbi.nlm.nih.gov/SAGE/sagexpsetup.cgi>) was utilised as the second datamining strategy for the identification of novel endothelial specific or preferentially endothelial genes. Two endothelial SAGE libraries (SAGE_Duke_HMVEC and SAGE_Duke_HMVEC+VEGF with a total of 110,790 sequences) were compared to twenty-four non-endothelial, cell line libraries (full list in Table 4, total of 733,461 sequences). Table 5 shows the status of expression of five known endothelial specific genes: von Willebrand's factor (vWF), two vascular endothelial growth factor receptors: fms-like tyrosine kinase 1 (flt1) and kinase insert domain receptor (KDR), tyrosine kinase receptor type tie (TIE1) and tyrosine kinase receptor type tek (TIE2/TEK), in these two SAGE pools.

Combined data gives highly accurate predictions

Twenty known genes were selected in the UniGene/EST screen (Table 6). These genes had no hits in the non-endothelial pool and at least one hit in the endothelial pool. The list contained at least four endothelial specific genes:

5 TIE1, TIE2/TEK, LYVE1 and multimerin, indicating ~20% accuracy of prediction. Other genes on the list, while certainly preferentially expressed in the endothelial cells, might not be endothelial specific. To improve on the prediction accuracy we decided to combine UniGene/EST screen with the xProfiler SAGE analysis. The xProfiler output consisted of a list of genes with

10 a ten times higher number of tags in the endothelial than in the non-endothelial pool sorted according to the certainty of prediction. A 90% certainty threshold was applied to this list. Table 7 shows how data from the two approaches were combined. Identity-level BLAST searches were performed on mRNAs (known genes) or phrap computed contigs (EST clusters representing novel genes) to

15 investigate how these genes were represented in the endothelial and non-endothelial pool. Subsequent experimental verification by RT-PCR (Figure 1) proved that the combined approach was 100% accurate, i.e. genes on the xProfiler list which had no matches the non-endothelial EST pool and at least one match in the endothelial pool were indeed endothelial specific.

20

DISCUSSION

There have been several reports of computer analysis of tissue transcriptomes. Usually an expression profile is constructed, based on the

25 number of tags assigned to a given gene or a class of genes (Bernstein *et al*, 1996, Welle *et al*, 1999, Bortoluzzi *et al*, 2000). An attempt can be made to identify tissue-specific transcripts, for example Vasmataz *et al*, (1997)

described three novel genes expressed exclusively in the prostate by *in silico* subtraction of libraries from the dbEST collection. Purpose made cDNA libraries may also be employed. Ten candidate granulocyte-specific genes have been identified by extensive sequence analysis of cDNA libraries derived
5 from granulocytes and eleven other tissue samples, namely a hepatocyte cell line, foetal liver, infant liver, adult liver, subcutaneous fat, visceral fat, lung, colonic mucosa, keratinocytes, cornea and retina (Itoh *et al*, 1998).

An analysis similar to the dbEST-based approach taken by Vasmataz *et al*, is
10 complicated by the fact that endothelial cells are present in all tissues of the body and endothelial-ESTs are contaminating all bulk tissue libraries. To validate this we used three well-known endothelial specific genes: KDR, FLT1, and TIE-2 as queries for BLAST searches against dbEST. Transcripts were present in a wide range of tissues with multiple hits in well vascularised
15 tissues (e.g. placenta, retina), embryonic (liver, spleen) or infant (brain) tissues. Additionally, we found that simple subtraction of endothelial EST libraries against all other dbEST libraries failed to identify any specific genes (data not shown).

20 Two very different types of expression data resources were used in our datamining efforts. The UniGene/EST screen was based on expressed sequence tag libraries from dbEST. There are 9 human endothelial libraries in the current release of dbEST with a relatively small total number of ESTs: ~11,117. Some well-known endothelial specific genes are not represented in
25 this dataset (Table 3). This limitation raised our concerns that genes with low levels of expression would be overlooked in our analysis. Therefore, we utilised another type of computable expression data: CGAP SAGE libraries.

SAGE tags are sometimes called small ESTs (usually 10-11 bp in length). Their major advantage is that they can be unambiguously located within the cDNA: they are immediately adjacent to the most 3' NlaIII restriction site. Though, there are only two endothelial CGAP SAGE libraries available at the moment, they contain an impressive total of ~111,000 tags - an approximately 10 times bigger dataset than the ~11,117 sequences in the endothelial EST pool. The combined approach proved very accurate (Table 8, Figure 1) when verified by RT-PCR.

10 We report here identification of two novel highly endothelial specific genes: endothelial cell-specific molecule 1 (ECSM1 – UniGene entry Hs.13957) and magic roundabout (UniGene entry Hs.111518). For a comprehensive summary of data available on these genes see Table 8.

15 Our combined datamining approach together with experimental verification is a powerful functional genomics tool. This type of analysis can be applied to many cell types not just endothelial cells. The challenge of identifying the function of discovered genes remains, but bioinformatics tools such as structural genomics, or homology and motif searches can offer insights that can
20 then be verified experimentally.

In summary, this screening approach has allowed the identification of novel endothelial cell specific genes and known genes whose expression was not known to be specific to endothelial cells. This identification both advances our
25 understanding of endothelial cell biology and provides new pharmaceutical targets for imaging, diagnosing and treating medical conditions involving the endothelium.

METHODS

PERL scripts

- 5 A number of PERL scripts were generated to facilitate large scale sequence retrieval, BLAST search submissions, and automatic BLAST output analysis.

Database sequence retrieval

- 10 Locally stored UniGene files (Build #111, release date May 2000) were used in the preparation of this report. The UniGene website can be accessed on the URL: www.ncbi.nlm.nih.gov/UniGene/, and UniGene files can be downloaded from the ftp repository: <ftp://ncbi.nlm.nih.gov/repository/unigene/>. Representative sequences for the human subset of UniGene (the longest EST
15 within the cluster) are stored in the file Hs.seq.uniq, while all ESTs belonging to the cluster are stored in a separate file called Hs.seq.

- Sequences were extracted from the dbEST database accessed locally at the HGMP centre using the Sequence Retrieval System (SRS version 5) getz
20 command. This was done repeatedly using a PERL script for all the libraries in the endothelial and non-endothelial subsets, and sequences were merged into two multiple-FASTA files.

Selection criteria for non-endothelial EST libraries

25

Selection of 108 non-endothelial dbEST libraries was largely manual. Initially the list of all available dbEST libraries

(http://www.ncbi.nlm.nih.gov/dbEST/libs_byorg.html) was searched using the keyword 'cells' and the phrase 'cell line'. While this searched identified most of the libraries, additional keywords had to be added for the list to be full: 'melanocyte', 'macrophage', 'HeLa', 'fibroblast'. In some cases, detailed
5 library description was consulted to confirm that library is derived from a cell line/primary culture. We also added a number of CGAP microdissected tumour libraries. For that, Library Browser (available at <http://www.ncbi.nlm.nih.gov/CGAP/hTGI/lbrow/cgaplb.cgi>) was used to search for the keyword 'microdissected'.

10

UniGene gene index screen

The UniGene gene transcript index was screened against the EST division of GenBank, dbEST. Both UniGene and dbEST were developed at the National
15 Centre for Biotechnology Information (NCBI). UniGene is a collection of EST clusters corresponding to putative unique genes. It currently consists of four datasets: human, mouse, rat and zebrafish. The human dataset is comprised of approximately 90,000 clusters (UniGene Build #111 May 2000). By means of very high stringency BLAST identity searches, we aimed to identify those
20 UniGene genes that have transcripts in the endothelial and not in the non-endothelial cell-type dbEST libraries. Throughout the project, University of Washington blast2 which is a gapped version was used as BLAST implementation. The E-value was set to 10e-20 in searches against the non-endothelial EST pool and to 10e-30 in searches against the smaller endothelial
25 pool.

While UniGene does not provide consensus sequences for its clusters, the longest sequence within the cluster is identified. Thus, this longest representative sequence (multiple FASTA file Hs.seq.uniq) was searched using very high stringency BLAST against the endothelial and non-endothelial EST pool. If such representative sequence reported no matches, the rest of the sequences belonging to the cluster (UniGene multiple-FASTA file Hs.seq) followed as BLAST queries. Finally, clusters with no matches in the non-endothelial pool and at least one match in the endothelial pool were selected using PERL scripts analysing BLAST textual output.

10

xProfiler SAGE subtraction

xProfiler enables an on-line user to perform a differential comparison of any combination of forty seven serial analysis of gene expression (SAGE) libraries with a total of ~2,300,000 SAGE tags using a dedicated statistical algorithm (Chen *et al*, 1998). xProfiler can be accessed on: <http://www.ncbi.nlm.nih.gov/SAGE/sagexpsetup.cgi>. SAGE itself is a quantitative expression technology in which genes are identified by typically a 10 or 11 bp sequence tag adjacent to the cDNA's most 3' NlaIII restriction site (Velculescu *et al*, 1995).

20

The two available endothelial cell libraries (SAGE_Duke_HMVEC and SAGE_Duke_HMVEC+VEGF) defined pool A and twenty-four (see Table 4 for list) non-endothelial libraries together built pool B. The approach was verified by establishing the status of expression of the five reference endothelial specific genes in the two SAGE pools (Table 5) using Gene to Tag Mapping (<http://www.ncbi.nlm.nih.gov/SAGE/SAGEcid.cgi>). Subsequently,

25

xProfiler was used to select genes differentially expressed between the pools A and B. The xProfiler output consisted of a list of genes with a ten fold difference in the number of tags in the endothelial compared to the non-endothelial pool sorted according to the certainty of prediction. A 90%
5 certainty threshold was applied to this list.

The other CGAP's on-line differential expression analysis tool, Digital Differential Display (DDD), relies on EST expression data (source library info) instead of using SAGE tags. We attempted to utilise this tool similarly to
10 SAGEmap xProfiler but have been unable to obtain useful results. Five out of nine endothelial and sixty-four out of hundred and eight non-endothelial cell libraries used in our BLAST-oriented approach were available for on-line analysis using DDD (<http://www.ncbi.nlm.nih.gov/CGAP/info/ddd.cgi>). When such analysis was performed the following were fifteen top scoring genes:
15 annexin A2, actin gamma 1, ribosomal protein large P0, plasminogen activator inhibitor type I, thymosin beta 4, peptidylprolyl isomerase A, ribosomal protein L13a, laminin receptor 1 (ribosomal protein SA), eukaryotic translation elongation factor 1 alpha 1, vimentin, ferritin heavy polypeptide, ribosomal protein L3, ribosomal protein S18, ribosomal protein L19, tumour protein
20 translationally-controlled 1. This list was rather surprising, did not include any well-known endothelial specific genes, did not have any overlap with SAGE results (Table 8), and contained many genes, that in the literature are reported to be ubiquitously expressed (ribosomal proteins, actin, vimentin, ferritin). A major advantage of our UniGene/EST screen is that instead of relying on
25 source library data and fallible EST clustering algorithms it actually performs identity-level BLAST comparisons in search of transcripts corresponding to a gene.

Mining data on UniGene clusters

To quickly access information about UniGene entries (e.g. literature references, STS sites, homologues, references to function) on-line resources were routinely used: NCBI's UniGene and LocusLink interfaces and Online Mendelian Inheritance in Man.

ESTs in UniGene clusters are not assembled into contigs, so before any sequence analysis, contigs were created using phrap assembler (for documentation on phrap see <http://bozeman.mbt.washington.edu/phrap.docs/phrap.html>).

To analyse genomic contig AC005795 (44,399) bp containing ECSM1, NIX Internet interface for multi-application analysis of large unknown nucleotide sequences was used. For further information on NIX see <http://www.hgmp.mrc.ac.uk/NIX/>. Alignment of ECSM1 against AC005795 was obtained using the NCBI interface to the Human Genome Interface: the NCBI Map Viewer. For further information on the NCBI Map Viewer see <http://www.ncbi.nlm.nih.gov/genome/guide/>.

To search for possible transmembrane domains and signal sequences in translated nucleotide sequences three Internet based applications were used: DAS <http://www.biokemi.su.se/~server/DAS/> (Cserzo *et al*, 1997), TopPred2 <http://www.biokemi.su.se/~server/toppred2/> (Heijne 1992), and SignalP <http://www.cbs.dtu.dk/services/SignalP/> (Nielsen *et al*, 1997).

PERL scripts

A number of PERL scripts were generated to facilitate large scale sequence retrieval, BLAST search submissions, and automatic BLAST output analysis.

5 Experimental verification

To experimentally verify specificity of expression we used the reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from three endothelial and seven non-endothelial cell types cultured *in vitro*.
10 Endothelial cultures were as follows: HMVEC (human microvascular endothelial cells), HUVEC (human umbilical vein endothelial cells) confluent culture and HUVEC proliferating culture. Non-endothelial cultures were as follows: normal endometrial stromal (NES) cells grown in normoxia and NES grown in hypoxia, MDA 453 and MDA 468 breast carcinoma cell lines, HeLa,
15 FEK4 fibroblasts cultured in normoxia and FEK4 fibroblasts cultured in hypoxia, and SW480, HCT116 - two colorectal epithelium cell lines.

If a sequence tagged site (STS) was available, dbSTS PCR primers were used and cycle conditions suggested in the dbSTS entry followed. Otherwise,
20 primers were designed using the Primer3 programme. Primers are listed in Table 9.

Tissue culture media, RNA extraction and cDNA synthesis

Cell-lines were cultured *in vitro* according to standard tissue culture protocols.
25 In particular, endothelial media were supplemented with ECGS (endothelial cell growth supplement - Sigma), and heparin (Sigma) to promote growth.

Total RNA was extracted using the RNeasy Minikit (Qiagen) and cDNA synthesised using the Reverse-IT 1st Strand Synthesis Kit (ABgene).

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10 285: 1553-1558.

Table 1.**Nine human endothelial libraries from dbEST**

Human aortic endothelium, 20 sequences, *in vitro* culture

Human endothelial cells, 346 sequences, primary isolate

Human endothelial cell (Y.Mitsui), 3 sequences, *in vitro* culture

Stratagene endothelial cell 937223, 7171 sequences, primary isolate

Aorta endothelial cells, 1245 sequences, primary isolate

Aorta endothelial cells, TNF treated, 1908 sequences, primary isolate

Umbilical vein endothelial cells I, 9 sequences

HDMEC cDNA library, 11 sequences, *in vitro* culture

Umbilical vein endothelial cells II, 404 sequences

Table 2.**Non-endothelial dbEST libraries.**

-
1. Activated T-cells I
 2. Activated T-cells II
 3. Activated T-cells III
 4. Activated T-cells IV
 5. Activated T-cells IX
 6. Activated T-cells V
 7. Activated T-cells VI
 8. Activated T-cells VII
 9. Activated T-cells VIII
 10. Activated T-cells X
 11. Activated T-cells XI
 12. Activated T-cells XII
 13. Activated T-cells XX
 14. CAMA1Ee cell line I
 15. CAMA1Ee cell line II
 16. CCRF-CEM cells, cyclohexamide treated I
 17. CdnA library of activated B cell line 3D5
 18. Chromosome 7 HeLa cDNA Library
 19. Colon carcinoma (Caco-2) cell line I
 20. Colon carcinoma (Caco-2) cell line II
 21. Colon carcinoma (HCC) cell line
 22. Colon carcinoma (HCC) cell line II
 23. HCC cell line (matastasis to liver in mouse)
 24. HCC cell line (matastasis to liver in mouse) II
 25. HeLa cDNA (T.Noma)
 26. HeLa SRIG (Synthetic retinoids induced genes)
 27. Homo sapiens monocyte-derived macrophages
 28. HSC172 cells I
 29. HSC172 cells II
-

-
30. Human 23132 gastric carcinoma cell line
 31. Human breast cancer cell line Bcap 37
 32. Human cell line A431 subclone
 33. Human cell line AGZY-83a
 34. Human cell line PCI-O6A
 35. Human cell line PCI-O6B
 36. Human cell line SK-N-MC
 37. Human cell line TF-1 (D.L.Ma)
 38. Human exocervical cells (CGLee)
 39. Human fibrosarcoma cell line HT1080
 40. Human fibrosarcoma cell line HT1080-6TGc5
 41. Human gastric cancer SGC-7901 cell line
 42. Human GM-CSF-deprived TF-1 cell line (Liu,Hongtao)
 43. Human HeLa (Y.Wang)
 44. Human HeLa cells (M.Lovett)
 45. Human Jurkat cell line mRNA (Thiele,K.)
 46. Human K562 erythroleukemic cells
 47. Human lung cancer cell line A549.A549
 48. Human nasopharyngeal carcinoma cell line HNE1
 49. Human neuroblastoma SK-ER3 cells (M.Garnier)
 50. Human newborn melanocytes (T.Vogt)
 51. Human pancreatic cancer cell line Patu 8988t
 52. Human primary melanocytes mRNA (I.M.Eisenbarth)
 53. Human promyelocytic HL60 cell line (S.Herblot)
 54. Human retina cell line ARPE-19
 55. Human salivary gland cell line HSG
 56. Human White blood cells
-

-
- 57. Jurkat T-cells I
 - 58. Jurkat T-cells II
 - 59. Jurkat T-cells III
 - 60. Jurkat T-cells V
 - 61. Jurkat T-cells VI
 - 62. Liver HepG2 cell line.
 - 63. LNCAP cells I
 - 64. Macrophage I
 - 65. Macrophage II
 - 66. Macrophage, subtracted (total
CdNA)
 - 67. MCF7 cell line
 - 68. Namalwa B cells I
 - 69. Namalwa B cells II
 - 70. NCI_CGAP_Br4
 - 71. NCI_CGAP_Br5
 - 72. NCI_CGAP_CLL1
 - 73. NCI_CGAP_GCB0
 - 74. NCI_CGAP_GCB1
 - 75. NCI_CGAP_HN1
 - 76. NCI_CGAP_HN3
 - 77. NCI_CGAP_HN4
 - 78. NCI_CGAP_HSC1
 - 79. NCI_CGAP_Li1
 - 80. NCI_CGAP_Li2
 - 81. NCI_CGAP_Ov5
 - 82. NCI_CGAP_Ov6
 - 83. NCI_CGAP_Pr1
 - 84. NCI_CGAP_Pr10
 - 85. NCI_CGAP_Pr11
 - 86. NCI_CGAP_Pr16
 - 87. NCI_CGAP_Pr18
 - 88. NCI_CGAP_Pr2
 - 89. NCI_CGAP_Pr20
 - 90. NCI_CGAP_Pr24
 - 91. NCI_CGAP_Pr25
 - 92. NCI_CGAP_Pr3
 - 93. NCI_CGAP_Pr4
 - 94. NCI_CGAP_Pr4.1
 - 95. NCI_CGAP_Pr5
 - 96. NCI_CGAP_Pr6
-

-
- 97. NCI_CGAP_Pr7
 - 98. NCI_CGAP_Pr8
 - 99. NCI_CGAP_Pr9
 - 100. Normal Human Trabecular Bone Cells
 - 101. Raji cells, cyclohexamide treated I
 - 102. Retinal pigment epithelium 0041 cell line
 - 103. Retinoid treated HeLa cells
 - 104. Soares melanocyte 2NbHM
 - 105. Soares_senescent_fibroblasts_Nb HSF
 - 106. Stratagene HeLa cell s3 937216
 - 107. Supt cells
 - 108. T, Human adult Rhabdomyosarcoma cell-line
-

Table 3.

Five genes known to be endothelial specific genes in the dbEST pools.

The number of ESTs in the endothelial pool is relatively small (~11,117) and not all known endothelial genes are represented

5

Known endothelial specific gene	Hits in the non- endothelial pool	Hits in the endothelial pool
von Willebrand factor (vWF)	1	27
flt1 VEGF receptor	---	---
KDR VEGF receptor	1	---
TIE1 tyrosine kinase	---	5
TIE2/TEK tyrosine kinase	---	2

Table 4.**Twenty-four non-endothelial cell SAGE-CGAP libraries.**

SYMBOL	DESCRIPTION
SAGE_HCT116	Colon, cell line derived from colorectal carcinoma
SAGE_Caco_2	Colon, colorectal carcinoma cell line
SAGE_Duke_H392	Brain, Duke glioblastoma multiforme cell line
SAGE_SW837	Colon, cancer cell line
SAGE_RKO	Colon, cancer cell line
SAGE_NHA(5th)	Brain, normal human astrocyte cells harvested at passage 5
SAGE_ES2-1	Ovarian Clear cell carcinoma cell line ES-2, poorly differentiated
SAGE_OVCA432-2	Ovary, carcinoma cell line OVCA432
SAGE_OV1063-3	Ovary, carcinoma cell line OV1063
SAGE_Duke_mhh-1	Brain, c-myc negative medulloblastoma cell line mhh-1
SAGE_Duke_H341	Brain, c-myc positive medulloblastoma cell line H341
SAGE_HOSE_4	Ovary, normal surface epithelium
SAGE_OVP-5	Ovary, pooled cancer cell lines
SAGE_LNCaP	Prostate, cell line. Androgen dependent
SAGE_HMEC-B41	Cell culture HMEC-B41 of normal human mammary epithelial cells
SAGE_MDA453	Cell line MDA-MB-453 of human breast carcinoma

SAGE_SKBR3	ATCC cell line SK-BR-3. Human breast adenocarcinoma
SAGE_A2780-9	Ovary, ovarian cancer cell line A2780
SAGE_Duke_H247_nor mal	Brain, glioblastoma multiforme cell line, H247
AGE_Duke_H247_Hyp oxia	Brain, Duke glioblastoma multiforme cell line, H247, grown under 1.5% oxygen
SAGE_Duke_post_crisi s_fibroblasts	Skin, post-crisis survival fibroblast cell-line
SAGE_Duke_precrisis_ fibroblasts	Skin, large T antigen transformed human fibroblasts clones
SAGE_A	Prostate, cancer cell line. Induced with synthetic androgen
SAGE_IOSE29-11	Ovary, surface epithelium line

Table 5.

Five known endothelial specific genes in the CGAP SAGE pools. TIE1 and TIE2/TEK have multiple hits in the non-endothelial pool (most in normal or carcinoma cell lines of ovarian origin). vWF is most endothelial specific
 5 having 80 hits in the endothelial pool and only one hit in the non-endothelial pool.

Known endothelial specific gene	Tags in the non-endothelial sage libraries	Tags in the endothelial sage libraries
von Willebrand factor (VWF)	1 (colon carcinoma cell line)	80
flt1 VEGF receptor	---	---
KDR VEGF receptor	1 (IOSE29 ovarian surface epithelium cell line)	6
TIE1 tyrosine kinase	17 (ovarian tumour and normal ovarian epithelium cell lines)	27
TIE2/TEK tyrosine kinase	4 (ovarian carcinoma and glioblastoma multiforme cell lines)	2

Table 6.

Results of the UniGene/EST screen. Twenty known genes were selected in the UniGene/EST screen (no hits in the non-endothelial pool and minimum one hit in the endothelial pool). At least four of these genes are known endothelial specific genes: TIE1, TIE2/TEK, LYVE1 and multimerin, indicating ~ 20 % prediction accuracy. Other genes, while certainly preferentially expressed in the endothelial cells, may not be endothelial specific.

Description	UniGene ID	Endothelial hits
TIE1 receptor endothelial tyrosine kinase	Hs.78824	5
Cytosolic phospholipase A2; involved in the metabolism of eicosanoids	Hs.211587	3
Branched chain alpha-ketoacid dehydrogenase	Hs.1265	2
CGMP-dependent protein kinase; cloned from aorta cDNA, strongly expressed in well vascularised tissues like aorta, heart, and uterus (Tamura <i>et al</i> , 1996)	Hs.2689	2
Lymphatic vessel endothelial hyaluronan receptor 1 - LYVE1 (Banerji <i>et al</i> , 1999)	Hs.17917	2
TRAF interacting protein: TNF signalling pathway	Hs.21254	2
Multimerin: a very big endothelial specific protein; binds platelet factor V, can also be found in platelets (Hayward <i>et al</i> , 1996)	Hs.32934	2
Diubiquitin (a member of the ubiquitin family); reported in dendritic and B lymphocyte cells;	Hs.44532	2

involved in antigen processing; this is first evidence that it is also present in endothelial cells (Bates <i>et al</i> , 1997)		
Beta-transducin family protein; also a homolog of D. melanogaster gene notchless: a novel WD40 repeat containing protein that modulates Notch signalling activity	Hs.85570	2
TIE2/TEK receptor endothelial tyrosine kinase	Hs.89640	2
BCL2 associated X protein (BAX)	Hs.159428	2
Sepiapterin reductase mRNA	Hs.160100	2
Retinoic acid receptor beta (RARβ)	Hs.171495	2
ST2 receptor: a homolog of the interleukin 1 receptor	Hs.66	1
Mitogen activated protein kinase 8 (MAPK8)	Hs.859	1
ERG gene related to the ETS oncogene	Hs.45514	1
PP35 similar to <i>E. coli</i> yhdg and R. Capsulatus nifR3	Hs.97627	1
Interphotoreceptor matrix proteoglycan; strongly expressed in retina and umbilical cord vein (Felbor <i>et al</i> , 1998)	Hs.129882	1
Methylmalonate semialdehyde dehydrogenase gene,	Hs.170008	1
HTLV-I related endogenous retroviral sequence	Hs.247963	1

Table 7.

xProfiler differential analysis was combined with data from the UniGene/EST screen achieving 100% certainty of prediction. xProfiler's output lists genes with 10-times higher number of tags in the endothelial than in the non-endothelial pool of SAGE-CGAP libraries. Hits corresponding to these genes in the endothelial and non-endothelial EST pools were identified by identity-level BLAST searches for mRNA (known genes) or phrap computed contig sequences (EST clusters representing novel genes). Genes are sorted according to the number of hits in the non-endothelial EST pool.

Known and predicted novel endothelial specific genes are in bold.

Unigene ID	Gene description	X profiler prediction certainty	Hits in endothelial EST pool	Hits in non-endothelial EST pool
Hs.13957	ESTs – ECSM1	97%	4	0
Hs.111518	magic roundabout, distant homology to human roundabout 1	100%	4	0
Hs.268107	multimerin	92%	5	0
Hs.155106	calcitonin receptor-like receptor activity modifying protein 2	97%	0	0
Hs.233955	ESTs	96%	0	0
Hs.26530	serum deprivation response (phosphatidylserine-	94%	3	1

	binding protein)			
Hs.83213	fatty acid binding protein 4	100%	3	1
Hs.110802	von Willebrand factor	100%	25	1
Hs.76206	cadherin 5, VE- cadherin (vascular endothelium)	100%	4	1
Hs.2271	endothelin 1	98%	9	2
Hs.119129	collagen, type IV, alpha 1	100%	4	6
Hs.78146	platelet/endothelial cell adhesion molecule (CD31 antigen)	99%	18	5
Hs.76224	EGF-containing fibulin-like extracellular matrix protein 1	100%	37	9
Hs.75511	connective tissue growth factor	100%	34	48

Table 8. Summary of available information on magic roundabout.

UniGene cluster ID and size		Full-length cDNA	Longest ORF	Transmembrane segments, signal peptide	Mapping information Genomic context Genomic clones	Description
ECSM1	Hs.13957		103 aa		Genomic neighbour: Tropomyosin	
	1100 bp		confirmed with 5'RACE		dbSTS G26129 and G28043 Chr. 19 Gene Map 98: Marker SGC3470, Marker sSG3414, IntervalD19S425-D19S418 AC005945, AC005795 (partial identity)	
Magic roundabout	Hs.111518	Partial cDNA FLJ20798 fs, clone ADSU02031 (acc. AK000805)	417 aa	One transmembrane domain predicted by TopPred2 and DAS. No signal peptide detected in the available 417 aa ORF (SignalP) however the true protein product is very likely to be larger	Genomic neighbour: integral transmembrane protein 1 (ITIM1) dbSTS G14646 and G14937 Chr. 11, Gene Map 98: Marker SHGC-11739, Interval D11S1353-D11S93	468 aa region of homology to the cytoplasmic portion of the roundabout axon guidance protein family: human ROBO1, rat ROBO1 and mouse dunt1 (E=1.3e-09) ORF has no apparent up-stream limit. This and size comparison to ROBO1 (1651 aa) suggests that true protein is very likely to be much larger Possible alternative polyA sites: the cDNA clone from adipocyte tissue seems to be polyadenylated in a different position to the sequence from the UniGene contig
	2076 bp	1496 bp				

Table 9.

List of primers used in RT-PCR reactions. dbSTS primers were used if a UniGene entry contained a sequence tagged site (STS). Otherwise, primers were designed using the Primer3 programme.

Gene	Primers (sequence or GenBank Accession for the STS)
ECSM1 – Hs.13957	G26129
Magic roundabout - Hs.111518	G14937
calcitonin receptor-like receptor activity modifying 2	G26129
Hs.233955	G21261
fatty acid binding protein 4	5'-TGC AGC TTC CTT CTC ACC TT-3' 5'-TCA CAT CCC CAT TCA CAC TG-3'
von Willebrand factor	5'-TGT ACC ATG AGG TTC TCA ATG C-3' 5'-TTA TTG TGG GCT CAG AAG GG-3'
serum deprivation response protein	G21528
collagen, type IV, alpha 1	G07125
EGF-containing fibulin-like extracellular matrix protein 1	G06992
connective tissue growth factor	5'-CAA ATG CTT CCA GGT GAA AAA-3' 5'-CGT TCA AAG CAT GAA ATG GA-3'

5

Table 10.

ESTs belonging to ECSM1 contig sequence are as follows:

10 EST SEQUENCES(30)

- AI540508, cDNAcloneIMAGE:2209821, Uterus, 3'read, 2.1kb
 AI870175, cDNAcloneIMAGE:2424998, Uterus, 3'read, 1.7kb
 AI978643, cDNAcloneIMAGE:2491824, Uterus, 3'read, 1.3kb
 15 AI473856, cDNAcloneIMAGE:2044374, Lymph, 3'read
 AI037900, cDNAcloneIMAGE:1657707, Wholeembryo, 3'read, 1.2kb

- AI417620, cDNAcloneIMAGE:2115082, 3'read, 1.0kb
 AA147817, cDNAcloneIMAGE:590062, 3'read
 AA968592, cDNAcloneIMAGE:1578323, 3'read, 0.7kb
 AW474729, cDNAcloneIMAGE:2853635, Uterus, 3'read
 5 R02352, cDNAcloneIMAGE:124282, 3'read, 0.7kb
 R01889, cDNAcloneIMAGE:124485, 5'read, 0.7kb
 AA446606, cDNAcloneIMAGE:783693, Wholeembryo, 3'read
 R02456, cDNAcloneIMAGE:124282, 5'read, 0.7kb
 T72705, cDNAcloneIMAGE:108686, 5'read, 0.7kb
 10 R01890, cDNAcloneIMAGE:124485, 3'read, 0.7kb
 AA147925, cDNAcloneIMAGE:590014, 5'read
 AI131471, cDNAcloneIMAGE:1709098, Heart, 3'read, 0.6kb
 AA733177, cDNAclone399421, Heart, 3'read
 AI039489, cDNAcloneIMAGE:1658903, Wholeembryo, 3'read, 0.6kb
 15 AI128585, cDNAcloneIMAGE:1691245, Heart, 3'read, 0.6kb
 AI540506, cDNAcloneIMAGE:2209817, Uterus, 3'read, 0.6kb
 AA894832, cDNAcloneIMAGE:1502815, Kidney, 3'read, 0.5kb
 AW057578, cDNAcloneIMAGE:2553014, Pooled, 3'read, 0.3kb
 AA729975, cDNAcloneIMAGE:1257976, GermCell, 0.3kb
 20 AI131016, cDNAcloneIMAGE:1706622, Heart, 3'read, 0.2kb
 AA147965, cDNAcloneIMAGE:590062, 5'read
 AA446735, cDNAcloneIMAGE:783693, Wholeembryo, 5'read
 AA147867, cDNAcloneIMAGE:590014, 3'read
 AI497866, cDNAcloneIMAGE:2125892, Pooled, 3'read
 25 T72636, cDNAcloneIMAGE:108686, 3'read, 0.7kb

Table 11.**30 ESTs within the magic roundabout sequence:**

- EST sequences in magic roundabout (55):
 AI803963, cDNAcloneIMAGE:2069520, 3'read, 0.9kb
 W88669, cDNAcloneIMAGE:417844, 3'read, 0.7kb
 35 AI184863, cDNAcloneIMAGE:1565500, Pooled, 3'read, 0.6kb
 AA011319, cDNAcloneIMAGE:359779, Heart, 3'read, 0.6kb
 AA302765, cDNAcloneATCC:194652, Adipose, 3'read
 AI278949, cDNAcloneIMAGE:1912098, Colon, 3'read, 0.7kb
 AI265775, cDNAcloneIMAGE:2006542, Ovary, 3'read
 40 AA746200, cDNAcloneIMAGE:1324396, Kidney, 0.5kb
 N78762, cDNAcloneIMAGE:301290, Lung, 3'read

- AI352263, cDNAcloneIMAGE:1940638, Wholeembryo, 3'read, 0.6kb
AA630260, cDNAcloneIMAGE:854855, Lung, 3'read, 0.5kb
C20950, cDNAclone(no-name), 3'read
W88875, cDNAcloneIMAGE:417844, 5'read, 0.7kb
5 AA156022, cDNAcloneIMAGE:590120, 3'read
N93972, cDNAcloneIMAGE:309369, Lung, 3'read, 1.7kb
AI217602, cDNAcloneIMAGE: 1732380, Heart, 3'read, 0.5kb
AW294276, cDNAcloneIMAGE:2726'347, 3'read
AA010931, cDNAcloneIMAGE:359779, Heart, 5'read, 0.6kb
10 AA303624, cDNAcloneATCC:115215, Aorta, 5'read
AI366745, cDNAcloneIMAGE:1935056, 3'read, 0.5kb
AA327257, cDNAcloneATCC:127927, Colon, 5'read
C06489, cDNAclonehbc5849, Pancreas
BE218677, cDNAcloneIMAGE:3176164, lung, 3'read
15 AA335675, cDNAcloneATCC:137498, Testis, 5'read
R84975, cDNAcloneIMAGE:180552, Brain, 3'read, 2.1kb
AI926445, cDNAcloneIMAGE:2459442, Stomach, 3'read, 1.9kb
H61208, cDNAcloneIMAGE:236318, Ovary, 3'read, 1.9kb
AA335358, cDNAcloneATCC:137019, Testis, 5'read
20 AI129190, cDNAcloneIMAGE:1509564, Pooled, 3'read, 0.8kb
T59188, cDNAcloneIMAGE:74634, Spleen, 5'read, 0.8kb
T59150, cDNAcloneIMAGE:74634, Spleen, 3'read, 0.8kb
R53174, cDNAcloneIMAGE:154350, Breast, 5'read, 0.8kb
AA156150, cDNAcloneIMAGE:590120, 5'read
25 AA302509, cDNAcloneATCC:114727, Aorta, 5'read
R99429, cDNAcloneIMAGE:201985, 5'read, 2.4kb
AI813787, cDNAcloneIMAGE:2421627, Pancreas, 3'read, 1.2kb
H62113, cDNAcloneIMAGE:236316, Ovary, 5'read, 1.0kb
R16422, cDNAcloneIMAGE:129313, 5'read, 0.7kb
30 T48993, cDNAcloneIMAGE:70531, Placenta, 5'read, 0.6kb
T05694, cDNAcloneHFBDF13, Brain
R84531, cDNAcloneIMAGE:180104, Brain, 5'read, 2.2kb
AI903080, cDNAclone(no-name), breast
AI903083, cDNAclone(no-name), breast
35 AA302764, cDNAcloneATCC:194652, Adipose, 5'read
AA341407, cDNAcloneATCC:143064, Kidney, 5'read
W16503, cDNAcloneIMAGE:301194, Lung, 5'read
AW801246, cDNAclone(no-name), uterus
AW959183, cDNAclone(no-name)
40 R85924, cDNAcloneIMAGE:180104, Brain, 3'read, 2.2kb
AA358843, cDNAcloneATCC:162953, Lung, 5'read

- BE161769, cDNAclone(no-name), head-neck
W40341, cDNAcloneIMAGE:309369, Lung, 5'read, 1.7kb
AA876225, cDNAcloneIMAGE:1257188, GermCell, 3'read
R99441, cDNAcloneIMAGE:202009, 5'read, 2.3kb
5 W76132, cDNAcloneIMAGE:344982, Heart, 5'read, 1.4kb,

Table 12.

110 ESTs in the mouse magic roundabout cluster (Mm.27782)

- 10 AI427548, cDNAcloneIMAGE:521115, Muscle, 3'read
AV022394, cDNAclone1190026N09, 3'read
BB219221, cDNAcloneA530053H04, 3'read
AI604803, cDNAcloneIMAGE:388336, Embryo, 3'read
15 AI504730, cDNAcloneIMAGE:964027, Mammarygland, 3'read
AI430395, cDNAcloneIMAGE:388336, Embryo, 5'read
AI181963, cDNAcloneIMAGE:1451626, Liver, 3'read
AV020471, cDNAclone1190017N14, 3'read
BB219225, cDNAcloneA530053H12, 3'read
20 BB224304, cDNAcloneA530086A21, 3'read
BB527740, cDNAcloneD930042M18, 3'read
W66614, cDNAcloneIMAGE:388336, Embryo, 5'read
BB097630, cDNAclone9430060E21, 3'read
AI152731, cDNAcloneIMAGE:1478154, Uterus, 5'read
25 AW742708, cDNAcloneIMAGE:2780289, innerear,170pooled, 3'read
BB118169, cDNAclone9530064M17, 3'read
AI839154, cDNAcloneUI-M-AO0-ach-e-11-0-UI, 3'read
BB206388, cDNAcloneA430075J10, 3'read
BB381670, cDNAcloneC230015E01, 3'read
30 BB199721, cDNAcloneA430017A19, 3'read
AI593217, cDNAcloneIMAGE:1177959, Mammarygland, 3'read
BB219411, cDNAcloneA530054L01, 3'read
BB220744, cDNAcloneA530061M19, 3'read
BB220944, cDNAcloneA530062O22, 3'read
35 BB390078, cDNAcloneC230066L23, 3'read
BB220730, cDNAcloneA530061L13, 3'read
AI615527, cDNAcloneIMAGE:964027, Mammarygland, 5'read
AI882477, cDNAcloneIMAGE:1396822, Mammarygland, 5'read
AV025281, cDNAclone1200012D01, 3'read
40 BB470462, cDNAcloneD230033L23, 3'read
BB247620, cDNAcloneA730020G03, 3'read

- BB555377, cDNAcloneE330019B13, 3'read
BB512960, cDNAcloneD730043I21
BB400157, cDNAcloneC330017F17, 3'read
BB320465, cDNAcloneB230385O10, 3'read
5 BB105670, cDNAclone9430096H10, 3'read
BB441462, cDNAcloneD030027B11, 3'read
BB137530, cDNAclone9830142O07, 3'read
AA553155, cDNAcloneIMAGE:964027, Mammarygland, 5'read
BB319763, cDNAcloneB230382G07, 3'read
10 BB451051, cDNAcloneD130007I05, 3'read
BB504672, cDNAcloneD630049J11, 3'read
AI429453, cDNAcloneIMAGE:569122, Embryo, 3'read
BB190585, cDNAcloneA330062J23, 3'read
BB257082, cDNAcloneA730076M18, 3'read
15 BB386699, cDNAcloneC230047P06, 3'read
BB295814, cDNAcloneB130042A09, 3'read
BB450972, cDNAcloneD130007A22, 3'read
AA718562, cDNAcloneIMAGE:1177959, Mammarygland, 5'read
BB223775, cDNAcloneA530083K18, 3'read
20 AV020555, cDNAclone1190018G05, 3'read
BB226083, cDNAcloneA530095K11, 3'read
BB482105, cDNAcloneD430007O19, 3'read
BB381671, cDNAcloneC230015E02, 3'read
BB383758, cDNAcloneC230030C02, 3'read
25 BB257519, cDNAcloneA730080D13, 3'read
BB265667, cDNAcloneA830021I17, 3'read
BB254777, cDNAcloneA730063K20, 3'read
AV240775, cDNAclone4732443F15, 3'read
BB315010, cDNAcloneB230352H04, 3'read
30 BB390074, cDNAcloneC230066L16, 3'read
BB517605, cDNAcloneD830025B17, 3'read
BB484410, cDNAcloneD430025H01, 3'read
BB357583, cDNAcloneC030022J01, 3'read
AV225639, cDNAclone3830431D12, 3'read
35 BB554921, cDNAcloneE330016A12, 3'read
BB161650, cDNAcloneA130061H21, 3'read
BB106720, cDNAclone9530002M22, 3'read
BB535465, cDNAcloneE030043P14, 3'read
BB357738, cDNAcloneC030024B10, 3'read
40 AV285588, cDNAclone5031411M12
BB188339, cDNAcloneA330048H22, 3'read

- AV337749, cDNAclone6430404F19, 3'read
BB065281, cDNAclone8030443H10, 3'read
BB148059, cDNAclone9930104N19, 3'read
AV252251, cDNAclone4833438P20, 3'read
5 BB184506, cDNAcloneA330012J24, 3'read
BB522445, cDNAcloneD930007M08, 3'read
BB520366, cDNAcloneD830041K23, 3'read
AV127290, cDNAclone2700047J01, 3'read
BB248651, cDNAcloneA730027F04, 3'read
10 BB008452, cDNAclone4732482M24, 3'read
BB550719, cDNAcloneE230024C07, 3'read
BB182033, cDNAcloneA230095N14, 3'read
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BB004855, cDNAclone4732463E03, 3'read
15 AV379748, cDNAclone9230013A19, 3'read
BB552137, cDNAcloneE230035B12, 3'read
BB288263, cDNAcloneIMAGE:3490042, mammary, 5'read
BB215681, cDNAcloneA530026M11, 3'read
BB251356, cDNAcloneA730046B16, 3'read
20 BB503441, cDNAcloneD630043F10, 3'read
BB500571, cDNAcloneD630029E03, 3'read
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BB098399, cDNAclone9430063L18, 3'read
25 BB213310, cDNAcloneA530009E09, 3'read
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BB214645, cDNAcloneA530021N22, 3'read
30 BB218254, cDNAcloneA530048K12, 3'read
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BB459759, cDNAcloneD130063G22, 3'read
BB485618, cDNAcloneD430032M09, 3'read
BB517699, cDNAcloneD830025J18, 3'read
35 BB535595, cDNAcloneE030044M09, 3'read
BB536291, cDNAcloneE030049D17, 3'read
BB552689, cDNAcloneE330001A16, 3'read
BB552709, cDNAcloneE33C001C16, 3'read

Example 2.**ECSM4 expression is restricted to endothelial cells.**

5 *In situ* hybridisation (ISH) of tumour and normal tissues showed that the expression of ECSM4 is restricted to vascular endothelial cells in adult angiogenic vessels only. Analysis of normal tissues showed that expression of ECSM4 is detected in human placenta and umbilical cord foetal tissue 10.8 weeks menstrual age. As shown in Figure 16, ECSM4 expression is highly
10 specific for the vascular endothelial cells of the blood vessel in placenta. Furthermore, expression was absent throughout a number of other normal tissues that were analysed, including adult liver, brain cerebrum and large vessels, prostate, colon, small bowel, heart, eye (choroid and sclera), ovary, stomach, breast and foetal bladder, testis, kidney (15.8 weeks) and foetal heart,
15 kidney, adrenal, intestine (11.3 weeks) foetal brain (10.6 weeks) and foetal eye (16.5 weeks) (data not shown).

ISH analysis of colorectal liver metastasis biopsies showed that expression of ECSM4 was restricted to vascular endothelial cells of the tumour vessels only
20 (Figure 17 and 18). No expression was detected in the surrounding normal tissue. Furthermore the enhanced expression in the vicinity of the necrotic tissues (Figure 18, necrotic tissue is indicated by the bright signal labelled *) is indicative and consistent with induction of ECSM4 expression by hypoxia. As such, ECSM4 may be a novel hypoxia regulated gene.

25

The highly restricted expression pattern of ECSM4 in angiogenic vessels in normal and tumour tissues in adult is entirely consistent with the endothelial

cell selective pattern of expression determined by the *in silico* analysis described in Example 1.

Methods

5 Blocks of formalin-fixed, paraffin-embedded tissues and tumours were obtained from the archives of the Imperial Cancer Research Fund Breast Pathology Group at Guys Hospital, London, UK. An antisense riboprobe to ECSM4 cDNA was prepared for specific localisation of the ECSM4 mRNA by *in situ* hybridisation. The methods for pretreatment, hybridisation, washing,
10 and dipping of slides in Ilford K5 for autoradiography has been described previously (Poulsom, R., Longcroft, J. M., Jeffrey, R. E., Rogers, L., and Steel, J. H. (1998) *Eur. J. Histochem.* **42**, 121-132). Films were exposed for 7 to 15 days before developing in Kodak D19 and counterstaining with Giemsa. Sections were examined under conventional or reflected light dark-field
15 conditions (Olympus BH2 with epi-illumination) under a x5, x10 or x20 objective that allowed individual auto-radiographic silver grains to be seen as bright objects on a dark background.

Example 3.

20 **ECSM4 polypeptide is detected only in endothelial cells.**

Antibodies capable of selectively binding the ECSM4 polypeptide were generated and used in immunohistochemistry to demonstrate the presence of ECSM4 polypeptide in a range of cell types (Figures 21 to 26). Tissue samples
25 were prepared by standard techniques in the art of immunohistochemistry.

Generation of antibodies recognising ECSM4.

The peptides MR 165, MR 311 and MR 336 were fused to Keyhole Limpet Haemocyanin (KLH) before immunisation of rabbits for production of polyclonal antibodies. The antibody MGO-5 was derived from rabbits
5 immunised with the peptide MR 165, whereas MGO-7 was derived from rabbits immunised with a mixture of MR 311 and MR 336. The sequence of the peptides used to generated the polyclonal antibodies is shown below with their reference within the amino acid sequence of full length human ECSM4 as shown in Figure 12.

10

MR 165 = LSQSPGAVPQALVAWRA (681-697)

MR 274 = DSVLTPEEVALCLEL (790-804)

MR 311 = TYGYISVPTA (827-836)

MR 336 = KGGVLLCPPRPCLTPT (852-867)

15

Example 4.

The magic roundabout EST sequence identified in the bioinformatics search for endothelial specific transcripts was used to isolate a cDNA of 3800 base pairs
20 in length from a human heart cDNA library. A screen using gene specific primers showed the gene to be present in libraries from heart, adult and foetal brain, liver, lung, kidney, muscle, placenta and small intestine but absent from peripheral blood leukocytes, spleen and testis. Highest expression was in the placental library. Comparison of the magic roundabout sequence to that of
25 roundabout revealed a transmembrane protein with homology throughout but absence of some extracellular domains. Thus, MR has two immunoglobulin and two fibronectin domains in the extracellular domain compared to five

immunoglobulin and two fibronectin domains in the extracellular domains of the neuronal specific roundabouts. A transmembrane domain was identified by (i) using the transmembrane predicting software PRED-TMR and (ii) using an alignment between human MR and human ROBO1 peptide sequences. Both
5 methods identified the same residues as the transmembrane region of human MR as amino acids 468-490. Thus, aa 1-467 are extracellular and aa 491-1007 are intracellular. The intracellular domain contains a putative proline rich region that is homologous to those in roundabout that are thought to couple to c-abl (Bashaw *et al* (2000) *Cell* 101: 703-715).

10

Human SHGC-11739 (GenBank acc. G14646) sequence tagged site (STS) was mapped to magic roundabout mRNA in a BLAST dbSTS search. This STS maps to chromosome 11 on the Stanford G3 physical map (region 5647.00 cR10000 LOD 1.09 bin 129). Nevertheless, much sequence is missing and the
15 genomic structure is not known. Search of the RIKEN database identified murine magic roundabout. The predicted molecular weight for the peptide core of human MR was 107,457 kDa. This was confirmed by *in vitro* translation (Figure 3).

20 Example 5.

ECSM4 expression is detectable in tumours

In situ hybridisation was used to characterise expression of ECSM4 *in vivo*.

25 Expression of ECSM4 was found to be very restricted (Table 13), with no signal detectable in many tissues including neuronal tissue. In contrast, strong expression was detected in placenta and a range of tumours including those of the brain, bladder and colonic metastasis to the liver (Figure 27). Expression

within tumours was restricted to the tumour vasculature. Immuno-histochemical staining of placenta confirmed endothelial specific expression of the protein.

- 5 A search of CGAP SAGE libraries for ECSM4 detected it only in endothelial and tumour libraries (Table 14). This was consistent with *in situ* hybridisation results in the adult showing that expression was restricted to tumour vessels (colon metastasis to liver, ganglioglioma, bladder and breast carcinoma).

10 **Table 13 Expression of magic roundabout in human tissue *in vivo*.**

Expression detected

Placenta and umbilical cord foetal tissue (10.8 weeks menstrual age)

- Vessels in colorectal liver metastasis, ganglioglioma, bladder and breast
15 carcinoma.

Expression not detected

Adult liver, brain cerebrum and large vessels, prostate, colon, small bowel, heart, eye choroid and sclera, ovary, stomach, breast

Table 14 CGAP SAGE libraries in which magic roundabout was found on the basis of gene to tag mapping

Library	Tags/million Tags
HDMEC	171
HDMEC + VEGF	224
Medulloblastoma	102
Glioblastoma multiforme	85
10 Ovary, serous adenocarcinoma	59
Glioblastoma multiforme, pooled	48

HDMEC, human dermal microvascular endothelial cells; VEGF, vascular endothelial growth factor.

Example 6.

Induction of ECSM4 in hypoxic endothelial cells

Initial RT-PCR detected ECSM4 expression in endothelial but not other cell lines such as fibroblasts (normal endometial and FEK4), colon carcinoma (SW480 and HCT116), breast carcinoma (MDA453 and MDA468) and HeLa cells. Ribonuclease protection analysis has confirmed and extended this (Figure 11a). ECSM4 expression was seen to be restricted to endothelium (three different isolates) and absent from fibroblast, carcinoma and neuronal cells. Induction of ECSM4 in hypoxia in endothelial (but not non-endothelial cells) was seen when expression of ECSM4 was analysed using two different RNase protection probes. Expression was on average 5.5 and 2.6 fold higher in

hypoxia for HUVEC and HDMEC respectively. Western analysis identified a weak band of 110kD in human dermal microvascular endothelial cells (HDMEC) but absent from the non-endothelial cells types (Figure 11b). The band was more intense when the HDMEC cells were exposed to 18 h hypoxia,
5 consistent with ECSM4 being a hypoxically regulated gene.

CLAIMS

1. A compound comprising (i) a moiety which selectively binds the polypeptide ECSM4 and (ii) a further moiety.
- 5 2. A compound comprising (i) a moiety which selectively binds the polypeptide ECSM1 and (ii) a further moiety.
3. A compound according to either one of Claims 1 or 2 wherein the moiety which selectively binds is an antibody.
- 10 4. A compound according to either one of Claims 1 or 2 wherein the moiety which selectively binds is a peptide.
- 15 5. A compound according to any one of Claims 1 to 4 wherein the further moiety is a readily detectable moiety.
6. A compound according to any one of Claims 1 to 4 wherein the further moiety is a directly or indirectly cytotoxic moiety.
- 20 7. A compound according to Claim 6 wherein the cytotoxic moiety is a directly cytotoxic chemotherapeutic agent.
8. A compound according to Claim 6 wherein the cytotoxic moiety is a directly cytotoxic polypeptide.
- 25

9. A compound according to Claim 6 wherein the cytotoxic moiety is a moiety which is able to convert a relatively non-toxic prodrug into a cytotoxic drug.
- 5 10. A compound according to Claim 6 wherein the cytotoxic moiety is a radiosensitizer.
11. A compound according to any one of Claims 1 to 4 wherein the further moiety comprises a nucleic acid molecule.
- 10 12. A compound according to Claim 11 wherein the nucleic acid molecule is a cytotoxic nucleic acid.
13. A compound according to Claim 12 wherein the nucleic acid molecule encodes a directly or indirectly cytotoxic polypeptide.
- 15 14. A compound according to Claim 12 wherein the nucleic acid molecule is directly cytotoxic.
- 20 15. A compound according to Claim 12 wherein the nucleic acid encodes a therapeutic polypeptide.
16. A compound according to Claim 7 wherein the cytotoxic moiety comprises a radioactive atom.

17. A compound according to Claim 16 wherein the radioactive atom is any one of phosphorus-32, iodine-125, iodine-131, indium-111, rhenium-186, rhenium-188 or yttrium-90.
- 5 18. A compound according to Claim 5 wherein the readily detectable moiety comprises a radioactive atom.
19. A compound according to Claim 18 wherein the radioactive atom is selected from any one of technitium-99m or iodine-123.
- 10 20. A compound according to Claim 5 wherein the readily detectable moiety comprises a suitable amount of any one of iodine-123, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.
- 15 21. A compound according to either one of Claims 1 or 2 wherein the further moiety is able to bind selectively to a directly or indirectly cytotoxic moiety.
- 20 22. A compound according to either one of Claims 1 or 2 wherein the further moiety is able to bind selectively to a readily detectable moiety.
23. A compound according to either one of Claims 1 or 2 wherein the selective binding moiety and the further moiety are polypeptides which
25 are fused.
24. A nucleic acid molecule encoding a compound according to Claim 23.

25. A method of imaging vascular endothelium in the body of an individual the method comprising administering to the individual an effective amount of a compound according to Claim 5.
- 5 26. A method according to Claim 25 wherein the vasculature is neovasculature.
- 10 27. A method of diagnosing or prognosing in an individual a condition which involves the vascular endothelium the method comprising administering to the individual an effective amount of a compound according to Claim 5.
- 15 28. A method according to Claim 27 further comprising the step of detecting the location of the compound in the individual.
29. A method according to any one of Claims 25 to 28 wherein the individual has cancer.
- 20 30. A method of treating an individual in need of treatment, the method comprising administering to the individual an effective amount of a compound according to any one of Claims 1 to 4 or 6 to 17.
- 25 31. A method according to Claim 30 wherein the individual in need of treatment has a proliferative disease or a condition involving the vascular endothelium such as any one of cancer, psoriasis, diabetic retinopathy, arteriosclerosis or menorrhagia.

32. A method of introducing genetic material selectively into vascular endothelial cells the method comprising contacting the cells with a compound according to any one of Claims 11-15.
- 5 .
33. Use of a compound according to Claim 5 in the manufacture of an agent for imaging the vasculature in a body of an individual.
34. Use of a compound according to Claim 5 in the manufacture of a
10 diagnostic or prognostic agent for a condition which involves the vascular endothelium.
35. Use of a compound according to any one of Claims 1 to 4 or 6 to 17 in the manufacture of a medicament for treating a condition involving the
15 vascular endothelium.
36. Use according to Claim 33 wherein the vasculature is cancer neovasculation or use according to either one of Claims 34 or 35 wherein the condition is cancer.
- 20
37. A polypeptide comprising or consisting of a fragment or variant or fusion of the ECSM4 polypeptide or a fusion of said fragment or variant provided that it is not a polypeptide consisting of the amino acid sequence given between residues 49 and 466 of Figure 4.
- 25
38. A polypeptide according to Claim 37 comprising or consisting of the sequence LSQSPGAVPQALVAWRA, DSVLTPEEVALCLEL,

TYGYISVPTA, KGGVLLCPRPCLTPT, WLADTW,
WLADTWRSTSGSRD, SPPTYGYIS,
GSLANGWGSASEDNAASARASLVSSSDGSFLAD or
FARALAVAVD or a fragment thereof of at least 5 amino acids.

5

39. A polypeptide according to Claim 37 comprising or consisting of a sequence present in the human ECSM4 but absent from the mouse ECSM4 sequence.

10 40. A polypeptide according to Claim 39 wherein the sequence consists or comprises the sequence GGDSL LGGRGSL, LLQPPARGHAHDGQALSTD L, EPQDYTEPVE, TAPGGQGAPWAE E or ERATQEPSEHGP.

15 41. A polypeptide comprising or consisting of the ECSM1 polypeptide or a fragment or variant or fusion thereof or a fusion of said fragment or variant.

20 42. A polynucleotide encoding a polypeptide according to any one of Claims 37 to 40, or the complement thereof or a polynucleotide which selectively hybridises to either of these which polynucleotide is not any one of the clone or cDNA corresponding to GenBank Accession No AK000805 or the ESTs whose GenBank Accession Nos are listed in Table 11 or Table 12.

25

43. A polynucleotide according to Claim 42 which encodes a polypeptide comprising an amino acid sequence with at least 65% identity to the amino acid sequence given in Figure 4 or Figure 7.
- 5 44. A polynucleotide encoding a polypeptide according to Claim 41 or the complement thereof or a polynucleotide which selectively hybridises to either of these provided that the polynucleotide is not one present in ATCC deposit No 209145 or the clone corresponding to GenBank Accession No AC011526 or the ESTs whose GenBank Accession Nos
10 are listed in Table 10.
45. A polynucleotide according to Claim 45 which encodes a polypeptide with at least 90% identity to the amino acid sequence given in Figure 2.
- 15 46. A polynucleotide according to any one of Claims 42 to 45 which is detectably labelled.
47. An expression vector comprising a polynucleotide according to any one of Claims 42 to 45.
20
48. A recombinant host cell comprising a polynucleotide according to any one of Claims 23, 41 to 45 or 47.
49. A recombinant host cell according to Claim 48 which is a bacterial cell.
25
50. A recombinant host cell according to Claim 48 which is a mammalian cell.

51. A method of producing a polypeptide according to any one of Claims 23 or 37 to 41 the method comprising expressing a polynucleotide according to any one of Claims 24, 42 to 45 or 47 or culturing a host cell according to any one of Claims 48 to 50.
52. An antibody capable of selectively binding to the ECSM4 polypeptide or the ECSM1 polypeptide.
53. An antibody according to Claim 52 which selectively binds a polypeptide comprising the amino acid sequence located between residues 1 and 467 of Figure 12.
54. An antibody according to Claim 53 which selectively binds any one of the amino acid sequences GGDSLLGGRGSL, LLQPPARGHAHDGQALSTD, EPQDYTEPVE, TAPGGQGAPWAE or ERATQEPSEHGP.
55. An antibody according to Claim 52 which selectively binds a polypeptide comprising the amino acid sequence located between residues 1 and 467 of Figure 12.
56. An antibody according to any one of Claims 52 to 54 which is a monoclonal antibody.
57. An antibody according to any one of Claims 52 to 55 which is detectably labelled.

58. A method of detecting endothelial damage or activation in an individual comprising obtaining a fluid sample from the individual and detecting the presence of fragments ECSM1 or ECSM4 in the sample.
- 5
59. A method of detecting a tumour or tumour neovasculature or cardiac disease or endometriosis or arteriosclerosis in an individual comprising obtaining a fluid sample from the individual and detecting the presence of fragments of ECSM1 or ECSM4 in the sample.
- 10
60. A method according to Claim 58 or 59 wherein the detection employs an antibody according to any one of Claims 52 to 55 or a compound according to any one of Claims 1 to 5, 18-20 or 22.
- 15
61. A method according to either one of Claims 58 or 60 wherein endothelial damage is diagnostic of cancer, cardiac disease, endometriosis or arteriosclerosis in the individual.
- 20
62. A method according to any one of Claims 57 to 61 wherein the individual is one receiving treatment for cancer, cardiac disease, endometriosis or arteriosclerosis and the amount of fragments of ECSM1 or ECSM4 in the sample is determined and compared either to that in a sample from an individual who does not have cancer, cardiac disease, endometriosis or arteriosclerosis and/or to the amount in a sample from the individual prior to commencement of said treatment and the comparison indicates the efficacy of treatment of the individual.
- 25

63. A method of modulating angiogenesis in an individual, the method comprising administering to the individual ECSM4, a peptide or fragment of ECSM4 or a ligand of ECSM4 or an antibody which selectively binds to ECSM4 or ECSM1.
- 5
64. A method of diagnosing a condition which involves aberrant or excessive growth of vascular endothelium in an individual comprising obtaining a sample containing nucleic acid from the individual and contacting said sample with a polynucleotide which selectively
10 hybridises to a nucleic acid which encodes the ECSM4 polypeptide or the ECSM4 polypeptide.
65. A method of reducing the expression of the ECSM4 or ECSM1 polynucleotide in an individual, comprising administering to the
15 individual an agent which selectively prevents expression of ECSM4 or ECSM1.
66. A method according to Claim 65 wherein the agent is an antisense nucleic acid.
- 20
67. A method of screening for a molecule that binds to ECSM4 or a suitable variant, fragment or fusion thereof, or a fusion of a said fragment or fusion thereof, the method comprising 1) contacting a) the said polypeptide with b) a test molecule, 2) detecting the presence of a
25 complex containing the ECSM4 polypeptide and a test molecule and optionally 3) identifying any test molecule bound to the ECSM4 polypeptide.

68. A polynucleotide comprising a promoter and/or regulatory portion of either of the ECSM1 or ECSM4 genes.
- 5 69. A polynucleotide according to Claim 68 which has transcriptional promoter activity selective to endothelial cells.
70. A polynucleotide according to either one of Claims 68 or 69 which is regulated by hypoxic conditions.
- 10 71. A polynucleotide as defined in any one of Claims 67 to 70 operatively linked to a polynucleotide encoding a polypeptide.
72. A polynucleotide according to Claim 71 wherein the polypeptide is a therapeutic polypeptide.
- 15 73. A polynucleotide according to Claim 72 wherein the polypeptide is a therapeutic polypeptide suitable for treating a hypoxic condition such as cancer, cardiac disease, endometriosis or arteriosclerosis.
- 20 74. A polynucleotide according to any one of Claims 68 to 73 which is suitable for use in gene therapy.
75. A kit of parts comprising a compound according to Claim 8 and a relatively non-toxic prodrug.
- 25

76. A kit of parts comprising a compound according to Claim 20 and any one of a directly or indirectly cytotoxic moiety or a readily detectable moiety to which the said compound is able to bind *via* its further moiety.
- 5 77. A compound according to any one of Claims 1 to 23, or a polypeptide according to Claims 37 to 41 or a polynucleotide according to any one of Claims 42 to 46 or 68 to 74 or an expression vector according to Claim 47 or an antibody according to Claims 52 to 56 for use in medicine.
- 10 78. A pharmaceutical composition comprising a polypeptide according to Claims 37 to 41 or a polynucleotide according to any one of Claims 42 to 46 or 68 to 74 or an expression vector according to Claim 47 or an antibody according to Claims 52 to 56 and a pharmaceutically acceptable carrier
- 15 79. A method of treating an individual with cancer, cardiac disease, a hypoxic condition, endometriosis or arteriosclerosis comprising administering to the individual a polynucleotide according to any one of
- 20 Claims 68 to 74.
80. A method of modulating angiogenesis in an individual comprising administering to the individual a polynucleotide according to any one of Claims 68 to 74 or a polynucleotide which is capable of expressing
- 25 ECSM4 or a fragment or variant thereof or which comprises an ECSM4 antisense nucleic acid.

81. A method according to Claim 80 when dependent on Claims 72 to 74 wherein the therapeutic polypeptide is any one or more of immunomodulatory, anti-cancer, a blood-clotting-influencing factor or an anti-proliferative or anti-inflammatory cytokine.
- 5
82. Use of a polynucleotide according to any one of Claims 68 to 74 in the manufacture of a medicament for treating cancer, cardiac disease, a hypoxic condition, endometriosis or arteriosclerosis.
- 10
83. Use of a polynucleotide according to any one of Claims 68 to 74 or a polynucleotide which is capable of expressing ECSM4 or a fragment or variant thereof or which comprises an ECSM4 antisense nucleic acid in the manufacture of a medicament for modulating angiogenesis.
- 15
84. A transgenic non-human mammal comprising a transgene which encodes a polypeptide according to any one of Claims 37 to 41.
85. A non-human mammal wherein if it contains an ECSM1 gene or an ECSM4 gene the gene or genes are missing or mutated.
- 20
86. A non-human mammal according to either one of Claims 84 or 85 which is a rodent, preferably mouse.

Figure 1

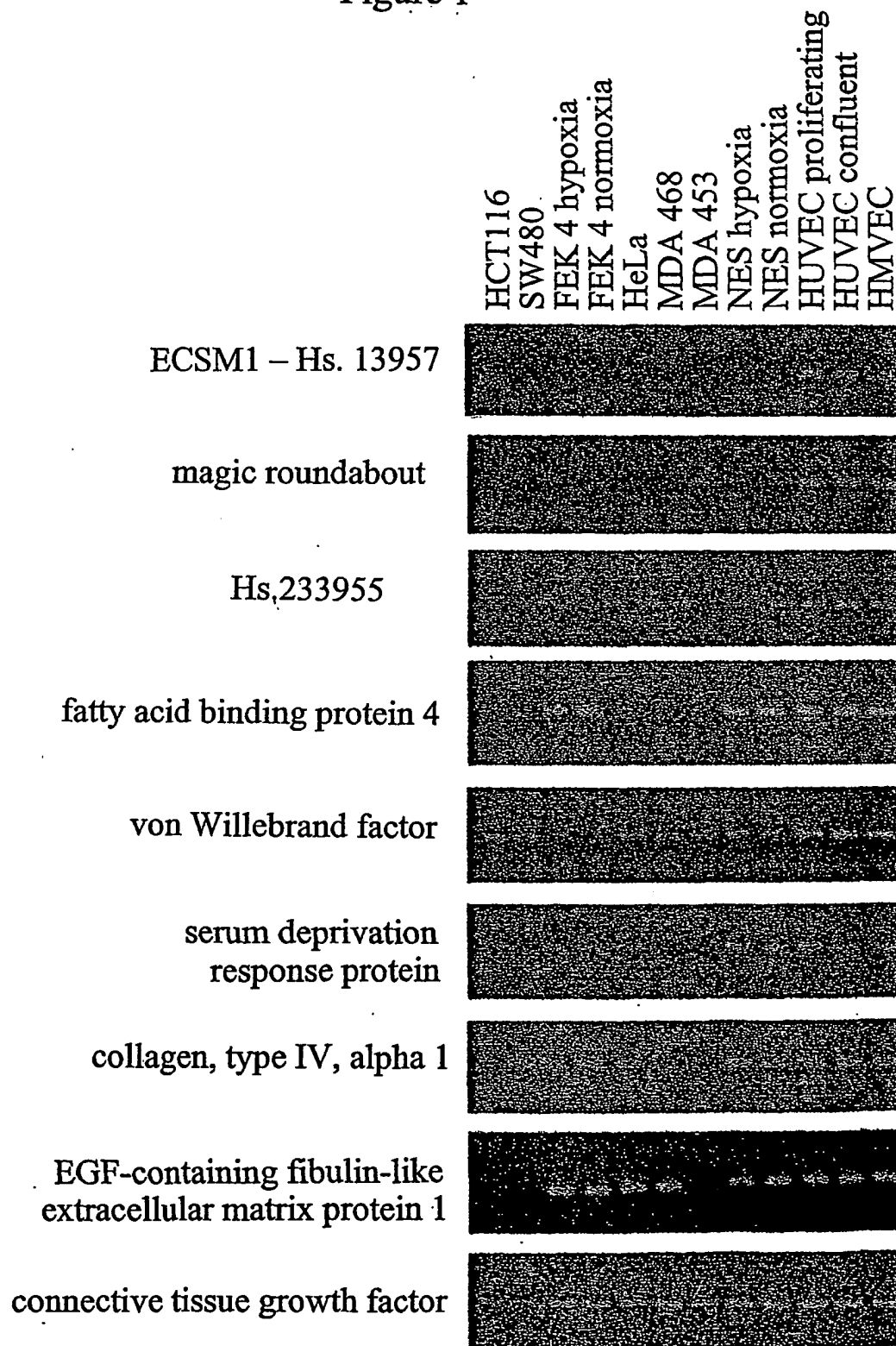


Figure 2 (page 1 of 2)

```
TGTCCTTATGCGGTGGCTCGCTGCTCAGAACAGGATGGCAGAGATGAGCACCACCATC
1  -----+-----+-----+-----+-----+-----+ 60

AAAAACTCAAGGACCAGTGCTGTGGGTCCAGTCATCTGTTTCATGGAATTCACCAGTCTG
61  -----+-----+-----+-----+-----+-----+ 120

GTATCTTCAAAATCCAGAAGGATGATGGCAGATGGCAGGAAGGAGGTAATCTG
121 -----+-----+-----+-----+-----+-----+ 180
           M A G R R K R V I W
GAAGAGTTTCCGGACCTACTCTGCTGTGTGATTAACAACACCAGGAAATTTTGATGA
181 -----+-----+-----+-----+-----+-----+ 240
K S F R T Y S A A V I K Q P P G N F D D
CACTGTCTCCTGAGCTCCTCCCTTCCTCGGGAAGAAAGCATTGAAACTACAAAAT
241 -----+-----+-----+-----+-----+-----+ 300
T V L L S S S L S S G K K S I E T T K I
AAAGTGTTATTGGCTGGAGTGAGGTCTCATGTCTGCTTATGCGGTGGCTCGCTGCTCAG
301 -----+-----+-----+-----+-----+-----+ 360
K C Y L A G V R S H V C L C G G S L L R
AACAGGGAACCATTTGGAGATACTACTTACTCTTTGAAGGCTTACAGTGAATGAATTCAA
361 -----+-----+-----+-----+-----+-----+ 420
T G N H W R Y S L L F E G L Q W N E F K
ATACGACTTATTGAGGAATTGAAGTTGACTTTATGGAGCTGATAAGAATCTTCTTTGGAG
421 -----+-----+-----+-----+-----+-----+ 480
Y D L F E E L K L T L W S *
AAAAAAGACTGGTACTTCTGAATTAACCAAAATCACAGTATTCTGAAGATGATTCTTACA
481 -----+-----+-----+-----+-----+-----+ 540
```

Figure 2 (page 2 of 2)

```

541  AAGCCTGCTGTTTCTACAAAGGCTGCTGATGATTCTACAAAGCCCTGCTAGTGTGGCT  600
      -----+-----+-----+-----+-----+-----+-----+
601  GTGGCCTCTGCTTAAAAAGTAGAAAAACACATTGATGCAGCATGTTACCCCCAACCTCCC
      -----+-----+-----+-----+-----+-----+-----+
661  TGCCTAAAGGCCCTCAGGGGCCCCCTCCTTGGGAAGAGGGAAGGGCGCGTGAGGATTGGTA  720
      -----+-----+-----+-----+-----+-----+-----+
721  AAGAGCCCGAATTAGGGGGGGGATGGGAGTGGTGGGAGAAATAAGGGGACACCTTCCATCCT  780
      -----+-----+-----+-----+-----+-----+-----+
781  TGGGATGCTCACCCCTGCCCAAATTGACCTTCCTGATGAAGGCCAGCTCCCAGAAAATGTG  840
      -----+-----+-----+-----+-----+-----+-----+
841  CCCTACAGTTACCTACTTTTCACCCCTAAACCCCTGCCCTTAGTCAAATCCTTTTCTTTT  900
      -----+-----+-----+-----+-----+-----+-----+
901  AAGCAATCAACTTCAATTCCCTTGATATAACCCCCAGTATAAAAGGGCTTTTATACCATTC  960
      -----+-----+-----+-----+-----+-----+-----+
961  ATCCTATTGCATGTAAGCCTTGGGTTTGGGAGGTAAACAGTGTGGGATTCCTCCCATTTTCAT  1020
      -----+-----+-----+-----+-----+-----+-----+
1021 TTCCCTGCCACCCAAACATGCCCTGTTTTTTTTTAAGCAATATTAATGTTTGTACTTCAG  1080
      -----+-----+-----+-----+-----+-----+-----+
      AAAAAAAAAAAAAAAAAAAAAA
1081 -----+-----+-----+ 1100

```

Figure 3

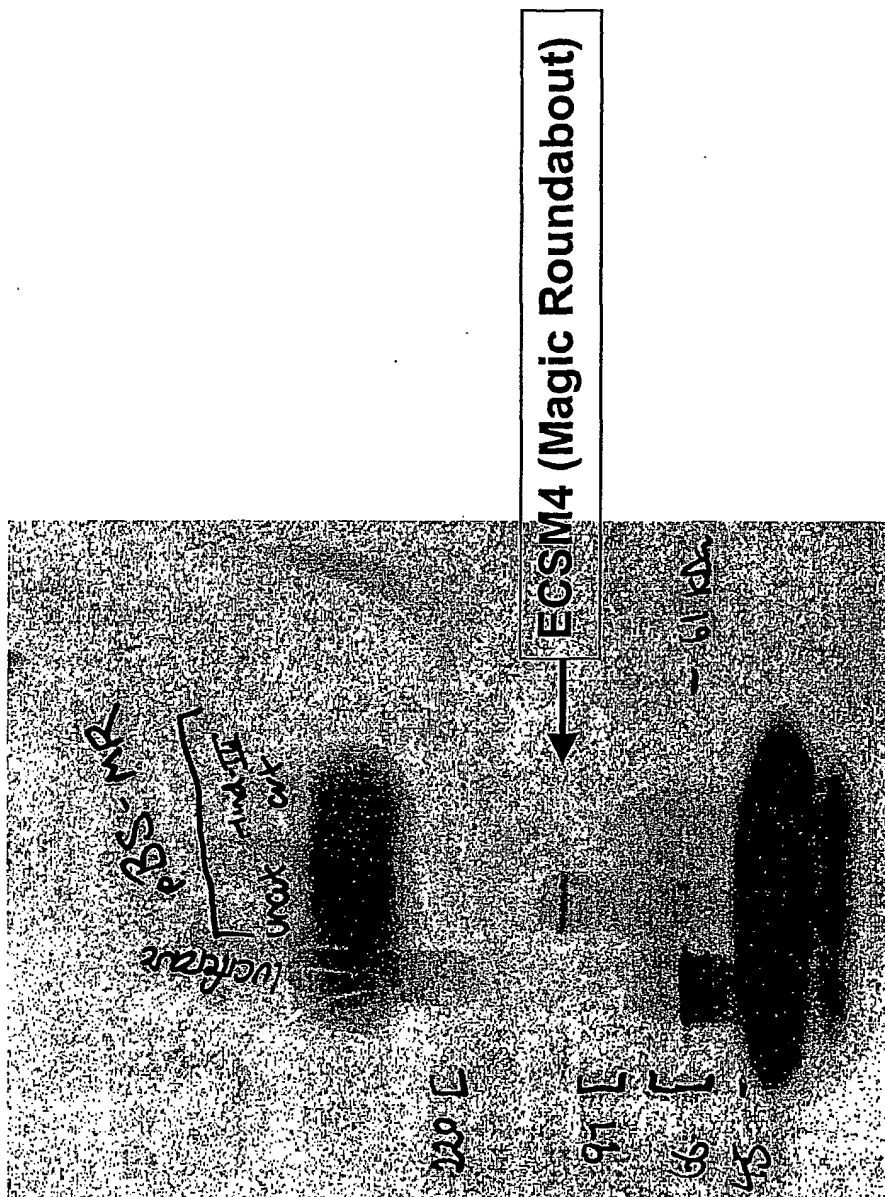


Figure 4 (page 1 of 3)

AACTGGTTGCGACACTGCGGTGTTGCACTCTGGCTGCTGCTTCTGGGCACCGCTGTGTGT
1 -----+-----+-----+-----+-----+-----+ 60
N W L R H C G V A L W L L L L G T A V C -
ATCCACCGCCGTCGCCGAGCTAGGGTGCTTCTGGGCCAGGTCTGTACAGATATACCAGT
61 -----+-----+-----+-----+-----+-----+ 120
I H R R R R A R V L L G P G L Y R Y T S -
GAGGATGCCATCCTAAACACAGGATGGATCACAGTGACTCCCAGTGTTGGCAGACACT
121 -----+-----+-----+-----+-----+-----+ 180
E D A I L K H R M D H S D S Q W L A D T -
TGGCGTTCCACCTCTGGCTCTCGGGACCTGAGCAGCAGCAGCAGCCTCAGCAGTCGGCTG
181 -----+-----+-----+-----+-----+-----+ 240
W R S T S G S R D L S S S S S L S S R L -
GGGGCGGATGCCCCGGGACCCACTAGACTGTCGTCGCTCCTTGCTCTCCTGGGACTCCCGA
241 -----+-----+-----+-----+-----+-----+ 300
G A D A R D P L D C R R S L L S W D S R -
AGCCCCGGCGTGCCCCCTGCTTCCAGACACCAGCACTTTTTATGGCTCCCTCATCGCTGAG
301 -----+-----+-----+-----+-----+-----+ 360
S P G V P L L P D T S T F Y G S L I A E -
CTGCCCTCCAGTACCCAGCCAGGCCAAGTCCCCAGGTCCCAGCTGTCAGGCGCCTCCCA
361 -----+-----+-----+-----+-----+-----+ 420
L P S S T P A R P S P Q V P A V R R L P -
CCCCAGCTGGCCCAGCTCTCCAGCCCCTGTTCCAGCTCAGACAGCCTCTGCAGCCGCAGG
421 -----+-----+-----+-----+-----+-----+ 480
P Q L A Q L S S P C S S S D S L C S R R -
GGACTCTCTTCTCCCCGCTTGTCTCTGGCCCCTGCAGAGGCTTGGAAGGCCAAAAAGAAG
481 -----+-----+-----+-----+-----+-----+ 540
G L S S P R L S L A P A E A W K A K K K -
CAGGAGCTGCAGCATGCCAACAGTTCCCCACTGCTCCGGGGCAGCCACTCCTTGAGCTC
541 -----+-----+-----+-----+-----+-----+ 600
Q E L Q H A N S S P L L R G S H S L E L -
CGGGCCTGTGAGTTAGGAAATAGAGGTTCCAAGAACCTTTCCAAAGCCCAGGGGCTGTG
601 -----+-----+-----+-----+-----+-----+ 660
R A C E L G N R G S K N L S Q S P G A V -

Figure 4 (page 2 of 3)

CCCCAAGCTCTGGTTGCCTGGCGGGCCCTGGGACCGAAACTCCTCAGCTCCTCAAATGAG
661 -----+-----+-----+-----+-----+-----+ 720
P Q A L V A W R A L G P K L L S S S N E -
CTGGTTACTCGTCATCTCCCTCCAGCACCCCTCTTTCTCATGAAACTCCCCCAACTCAG
721 -----+-----+-----+-----+-----+-----+ 780
L V T R H L P P A P L F P H E T P P T Q -
AGTCAACAGACCCAGCCTCCGGTGGCACCACAGGCTCCCTCCTCCATCCTGCTGCCAGCA
781 -----+-----+-----+-----+-----+-----+ 840
S Q Q T Q P P V A P Q A P S S I L L P A -
GCCCCCATCCCCATCCTTAGCCCCCTGCAGTCCCCCTAGCCCCCAGGCCTCTTCCCTCTCT
841 -----+-----+-----+-----+-----+-----+ 900
A P I P I L S P C S P P S P Q A S S L S -
GGCCCCAGCCCAGCTTCCAGTCGCCTGTCCAGCTCCTCACTGTCATCCCTGGGGGAGGAT
901 -----+-----+-----+-----+-----+-----+ 960
G P S P A S S R L S S S S L S S L G E D -
CAAGACAGCGTGCTGACCCCTGAGGAGGTAGCCCTGTGCTTGGAACTCAGTGAGGGTGAG
961 -----+-----+-----+-----+-----+-----+ 1020
Q D S V L T P E E V A L C L E L S E G E -
GAGACTCCCAGGAACAGCGTCTCTCCCATGCCAAGGGCTCCTTCACCCCCCACCACCTAT
1021 -----+-----+-----+-----+-----+-----+ 1080
E T P R N S V S P M P R A P S P P T T Y -
GGGTACATCAGCGTCCCAACAGCCTCAGAGTTCACGGACATGGGCAGGACTGGAGGAGGG
1081 -----+-----+-----+-----+-----+-----+ 1140
G Y I S V P T A S E F T D M G R T G G G -
GTGGGGCCCAAGGGGGAGTCTTGCTGTGCCCACCTCGGCCCTGCCTCACCCCCACCCCC
1141 -----+-----+-----+-----+-----+-----+ 1200
V G P K G G V L L C P P R P C L T P T P -
AGCGAGGGCTCCTTAGCCAATGGTTGGGGCTCAGCCTCTGAGGACAATGCCGCCAGCGCC
1201 -----+-----+-----+-----+-----+-----+ 1260
S E G S L A N G W G S A S E D N A A S A -
AGAGCCAGCCTTGTCAGCTCCTCCGATGGCTCCTTCCTCGCTGATGCTCACTTTGCCCGG
1261 -----+-----+-----+-----+-----+-----+ 1320
R A S L V S S S D G S F L A D A H F A R -

Figure 4 (page 3 of 3)

```

GCCCTGGCAGTGGCTGTGGATAGCTTTGGTTTCGGTCTAGAGCCCAGGGAGGCAGACTGC
1321 -----+-----+-----+-----+-----+-----+ 1380
      A L A V A V D S F G F G L E P R E A D C -
      GTCTTCATAGGTATGTGAGGTCTCCCCATCTTACTCCTCACTCATGCCCTTGCCCTTTCT
1381 -----+-----+-----+-----+-----+-----+ 1440
      V F I G M * -
      AACAACTGTTATCATGTCATCATTTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1441 -----+-----+-----+-----+-----+-----+ 1496
```

Figure 5 (page 1 of 3)

ECSM4 Length: 2076

1 AGGGGACTCT CTTCTCCCCG CTTGTCTCTG GCCCCTGCAG AGGCTTGGAA
51 GGCCAAAAAG AAAGCAGGAG CTGCAGCATG CCAACAGTTC CCCACTGCTC
101 CGGGGCAGCC ACTCCTTAGA GCTCCGGGCC TGTGAGTTAG GAAATAGAGG
151 TTCCAAGAAC CTTTCCCAA GCCCAGGAGC TGTGCCCCAA GCTCTGGTTG
201 CCTGGCGGGC CCTGGGACCG AAACCTCCTCA GCTCCTCAAA TGAGCTGGTT
251 ACTCGTCATC TCCCTCCAGC ACCCCTCTTT CCTCATGAAA CTCCCCAAC
301 TCAGAGTCAA CAGACCCAGC CTCCGGTGGC ACCACAGGCT CCCTCCTCCA
351 TCCTGCTGCC AGCAGCCCCC ATCCCCATCC TTAGCCCCTG CAGTCCCCCT
401 AGCCCCCAGG CCTCTTCCCT CTCTGGCCCC AGCCCAGCTT CCAGTCGCCT
451 GTCCAGCTCC TCACTGTCAT CCCTGGGGGA GGATCAAGAC AGCGTGCTGA
501 CCCCTGAGGA GGTAGCCCTG TGCTTGGAAC TCAGTGAGGG TGAGGAGACT
551 CCCAGGAACA GCGTCTCTCC CATGCCAAGG GTTCCTTCAC CCCCCACCAC
601 CTATGGGTAC ATCAGCGTCC CAACAGCCTC AGAGTTCACG GACATGGGCA
651 GGACTGGAGG AGGGGTGGGG CCCAAGGGGG GAGTCTTGCT GTGCCACCT
701 CGGCCCTGCC TCACCCCCAC CCCCAGCGAG GGCTCCTTAG CCAATGGTTG
751 GGGCTCAGCC TCTGAGGACA ATGCCGCCAG CGCCAGAGCC AGCCTTGTCA
801 GCTCCTCCGA TGGCTCCTTC CTCGCTGATG CTCACTTTGC CCGGGCCCTG
851 GCAGTGGCTG TGGATAGCTT TGGTTTCGGT CTAGAGCCCA GGGAGGCAGA
901 CTGCGTCTTC ATAGATGCCT CATCACCTCC CTCCCCACGG GATTGAGATC
951 TTCCTGACCC CCAACCTCTC CCTGCCCCCTG TGGGAAGTGG AGGCCAGACT
1001 GGTGGAAGA CAATGGAAGG TCAGCCACAC CCAGCGGCTG GGAAGGGGGA
1051 TGCTCCCTG GCCCCCTGAC TCTCAGATCT CTTCCAGAG AAGTCAGCTC
1101 CACTGTCGTA TGCCCAAGGG TGGGTGCTTC TCCTGTAGAT TACTCCTGAA
1151 CCGTGTCCCT GAGACTTCCC AGACGGGAAT CAGAACCACT TCTCCTGTCC
1201 ACCACAAGA CCTGGGCTGT GGTGTGTGGG TCTTGGCCTG TGTTTCTCTG
1251 CAGCTGGGGT CCACCTTCCC AAGCCTCCAG AGAGTTCTCC CTCCACGATT
1301 GTGAAAACAA ATGAAAACAA AATTAGAGCA AAGCTGTACC TGGGAGCCCT

Figure 5 (page 2 of 3)

1351 CAGGGAGCAA AACATCATCT CCACCTGACT CCTAGCCACT GCTTTCTCCT
1401 CTGTGCCATC CACTCCCACC ACCCAGGTG TTTTGGCCT GAAGGAGCAA
1451 GCCCTGCCTG CTGGCTTTTC CCCCCAACCA TTTGGGATTC ACAGGGAAGT
1501 GGGAGGGAGC CCAGAGGGTG GCCTTTTGTG GGAGGGACAG CAGTGGCTGC
1551 TGGGGGAGAG GGCTGTGGAG GAAGGAGCTT CTCGGAGCCC CCTCTCAGCC
1601 TTACCTGGGC CCTCCTCTA GAGAAGAGCT CAACTCTCTC CCAACCCTCA
1651 CCAATGGAAA GAAAATAATT ATGAATGCCG ACTGAGGCAC TGAGGCCCCCT
1701 ACCTCATGCC CAAAACAAAG GGGTTCAAGG CTGGGTCTAG CGAGGATGCT
1751 TGAAGGAAGG GAGGTATGGA GCCCGTAGGT CAAAAGCACC CATCCTCGTA
1801 CTGTTGTCAC TATGAGCTTA AGAAATTTGA TACCATAAAA TGGTAAAGAC
1851 TTGAGTTCTG TGAGATCATT CCCCGGAGCA CCATTTT TAG GGGAGCACCT
1901 GGAGAGATGG CAAGAATTTCTGAGTTAGG CAGGGATCAG GCATTCATTG
1951 ACACTCAGGG AGTGTCACAC ATTTCTGTTC TGCAATTAAA GGGAGAATGA
2001 GGTTTCATCCA CCAAATTTTA AGCAGAATAT AGGAAGGGCA GGGGTGGGGA
2051 GTTTCAGGGT CTGCTGGTCC TGGGCA

Figure 5 (page 3 of 3)

START: 2 STOP: 948

Translation:

Length: 315

1 GDSLLPACIW PLQRLGRPKE KQELQHANS PLLRGSHSLE LRACELGNRG
51 SKNLSQSPGA VPQALVAWRA LGPKLLSSSN ELVTRHLPPA PLFPHETPPT
101 QSQQTQPPVA PQAPSSILLP AAPIPILSPC SPPSPQASSL SGPSPASSRL
151 SSSSLSSLGE DQDSVLTPEE VALCLELSEG EETPRNSVSP MPRVPSPPTT
201 YGYISVPTAS EFTDMGRTGG GVGPKGGVLL C`PPRPCLTPT PSEGSLANGW
251 GSASEDNAAS ARASLVSSSD GSFLADAHFA RALAVAVDSF GFGLEPREAD
301 CVFIDASSPP SPRD*

Figure 6 (page 1 of 2)

Gap Weight: 50 Average Match: 10.000
 Length Weight: 3 Average Mismatch: 0.000

Quality: 9397 Length: 2553
 Ratio: 6.281 Gaps: 1
 Percent Similarity: 92.738 Percent Identity: 92.738

Match display thresholds for the alignment(s):

| = IDENTITY
 : = 5
 . = 1

magic.seq x hs.111518.rev September 13, 2000 14:21 ..

```

      .
      .
451 TCCAGCTCAGACAGCCTCTGCAGCCGCGAGGGGACTCTCTTCTCCCCGCTT 500
      |||||
1  .....AGGGGACTCTCTTCTCCCCGCTT 23

501 GTCTCTGGCCCCTGCAGAGGCTTGGAAGGCCAAAAAG.AAGCAGGAGCTG 549
      |||||
24 GTCTCTGGCCCCTGCAGAGGCTTGGAAGGCCAAAAAGAAAGCAGGAGCTG 73

550 CAGCATGCCAACAGTTCCCCACTGCTCCGGGGCAGCCACTCCTTGGAGCT 599
      |||||
74 CAGCATGCCAACAGTTCCCCACTGCTCCGGGGCAGCCACTCCTTAGAGCT 123

600 CCGGGCCTGTGAGTTAGGAAATAGAGGTTCCAAGAACCTTTCCCAAAGCC 649
      |||||
124 CCGGGCCTGTGAGTTAGGAAATAGAGGTTCCAAGAACCTTTCCCAAAGCC 173

650 CAGGGGCTGTGCCCCAAGCTCTGGTTGCCTGGCGGGCCCTGGGACCGAAA 699
      |||||
174 CAGGAGCTGTGCCCCAAGCTCTGGTTGCCTGGCGGGCCCTGGGACCGAAA 223

700 CTCCTCAGCTCCTCAAATGAGCTGGTTACTCGTCATCTCCCTCCAGCACC 749
      |||||
224 CTCCTCAGCTCCTCAAATGAGCTGGTTACTCGTCATCTCCCTCCAGCACC 273

750 CCTCTTTCCTCATGAAACTCCCCCAACTCAGAGTCAACAGACCCAGCCTC 799
      |||||
274 CCTCTTTCCTCATGAAACTCCCCCAACTCAGAGTCAACAGACCCAGCCTC 323

800 CGGTGGCACCACAGGCTCCCTCCTCCATCCTGCTGCCAGCAGCCCCCATC 849
      |||||
324 CGGTGGCACCACAGGCTCCCTCCTCCATCCTGCTGCCAGCAGCCCCCATC 373

850 CCCATCCTTAGCCCCCTGCAGTCCCCCTAGCCCCCAGGCCTCTTCCCTCTC 899
      |||||
374 CCCATCCTTAGCCCCCTGCAGTCCCCCTAGCCCCCAGGCCTCTTCCCTCTC 423

```

Figure 6 (page 2 of 2)

900 TGGCCCCAGCCCAGCTTCCAGTCGCCTGTCCAGCTCCTCACTGTCATCCC 949
|||
424 TGGCCCCAGCCCAGCTTCCAGTCGCCTGTCCAGCTCCTCACTGTCATCCC 473
|||
950 TGGGGGAGGATCAAGACAGCGTGCTGACCCCTGAGGAGGTAGCCCTGTGC 999
|||
474 TGGGGGAGGATCAAGACAGCGTGCTGACCCCTGAGGAGGTAGCCCTGTGC 523
|||
1000 TTGGAACCTCAGTGAGGGTGAGGAGACTCCCAGGAACAGCGTCTCTCCCAT 1049
|||
524 TTGGAACCTCAGTGAGGGTGAGGAGACTCCCAGGAACAGCGTCTCTCCCAT 573
|||
1050 GCCAAGGGCTCCTTCACCCCCACACCTATGGGTACATCAGCGTCCCAA 1099
|||
574 GCCAAGGGTTCCTTCACCCCCACACCTATGGGTACATCAGCGTCCCAA 623
|||
1100 CAGCCTCAGAGTTCACGGACATGGGCAGGACTGGAGGAGGGGTGGGGCCC 1149
|||
624 CAGCCTCAGAGTTCACGGACATGGGCAGGACTGGAGGAGGGGTGGGGCCC 673
|||
1150 AAGGGGGGAGTCTTGCTGTGCCACCTCGGCCCTGCCTCACCCCCACCCC 1199
|||
674 AAGGGGGGAGTCTTGCTGTGCCACCTCGGCCCTGCCTCACCCCCACCCC 723
|||
1200 CAGCGAGGGCTCCTTAGCCAATGGTTGGGGCTCAGCCTCTGAGGACAATG 1249
|||
724 CAGCGAGGGCTCCTTAGCCAATGGTTGGGGCTCAGCCTCTGAGGACAATG 773
|||
1250 CCGCCAGCGCCAGAGCCAGCCTTGTCAGCTCCTCCGATGGCTCCTTCCTC 1299
|||
774 CCGCCAGCGCCAGAGCCAGCCTTGTCAGCTCCTCCGATGGCTCCTTCCTC 823
|||
1300 GCTGATGCTCACTTTGCCCCGGGCCCTGGCAGTGGCTGTGGATAGCTTTGG 1349
|||
824 GCTGATGCTCACTTTGCCCCGGGCCCTGGCAGTGGCTGTGGATAGCTTTGG 873
|||
1350 TTTCGGTCTAGAGCCAGGGAGGCAGACTGCGTCTTCATAGGTATGTGAG 1399
|||
874 TTTCGGTCTAGAGCCAGGGAGGCAGACTGCGTCTTCATAGATGCCTCAT 923
|||
1400 GTCTCCCCATCTTACTCCTCACTCATGCCCTTGCCCTTCTAACAACCTGT 1449
|||
924 CACCTCCCTCCCCACGGGATTGAGATCTTCTGACCCCCAACCTCTCCCT 973
|||
1450 TATCATGTCTCATTTGTTAAAAA... 1496
|||
974 GCCCCTGTGGGAAGTGGAGGCCAGACTGGTTGGAAGACAATGGAAGGTCA 1023
|||

Figure 7 (page 1 of 2)

Mouse magic roundabout contig sequence:

Length: 1271

```
1  GGGTCTTTAC AGTTTATAG AATTAAGTTC CTTAAGCTCA GAGTGGGGGT
51  AGAAATGAGA ATAGGGAATT GGTCCCTGTG CTTCCCTGCGT CTTATCCTT
101 TCAGTCTCCT CCAATGATTT CACTTTGAAG GATTGAATGT GAGGCTGTAT
151 AGGGCCAGT GCATCCAGAA CGTTTCTCCA TAAGTTTCCT TGGATGGTTG
201 TGAATGGGGA AAGGGTTGAG TTGGTGTGTG AAGGGAGGAG TCCAAGTTAA
251 TATTAGAGGG GTCCTCCACA GGTCCACCAA CAGAGGCCCT CACCAAAAAA
301 CATTCTGTC CTTCTGAAG ACCTGGTTGG CTTCCCTTCT TTCCATGATC
351 CACTTAGCG GGAGCTCCGG AGCCAGGCTT ACTTAGGCCA AAGGTTCTGG
401 TTGTGGAGAG TCTGCTGTCC TGAAGATGCT GTCTTGTCTT CAGTGGGAAT
451 CCAAGACTCC CGTGATCATA TTTTGGTTTG CTTTCATTTA TTTTAACAAT
501 CCCAATGACA GAGCTCTCCA GAAGCCTAGT GACAGTGGAC TTCTATTACA
551 GAGAAGCATA GGCCAAGACC TCCACATGTG AGAAAGCCAG GGGACAGACA
601 GGAGAGTGGT CTGGTGCTC TTCTGGCCTT CTCAGGGACA ATCAGGAGG
651 AATCACACAG CCTTGGGCAC AGCACCAGTT AGCCAACCTC GCTGGGAAGA
```

Figure 7 (page 2 of 2)

701 GGCCTAGAA TCAGGAGGCC AGGAGGCAG CCCCTCCCC AGCCTCTGGG
751 TGTGGCTGAT CTCAGCATCT TCCAACCAGT CTGGCCTCCA CTCCACAAA
801 GGCAGAGAGA AGCTTCGGGT CAGGGAGAGA TCACCCCGAG GGGAGGGAGG
851 TGATGAGGCA TCAGTGAAGA CACAGTCAGC TTCCCTGGGA TCCAGACTGA
901 GGCCTAAGCT ATCCACAGCC ACTGCCAGGG CACGAGCAAA GTGAGTATCA
951 GCGAGGAAGG AGCCATCAGA AGAGCTAACC AGGCTGGCC TGGCGCTGGG
1001 GACATTGTCC TCAGAAAGCTG AGCCCCAACC ATTGGCCAGG GAGCCCTCGC
1051 TGGGTGTAGG GGTGGGCAG GGCCGAGGTG GATACAGTAA GTTCCCAACC
1101 TCAGACCCCA CGCCCCCGCC AGCTCTGCC ATGTCTGCCA GTCCCTGAGCA
1151 GGTTGGTATG CTGATATAGC CATAGGTTGT TGGCGGGGAA GGAGCTCTTG
1201 GCATAGGAGA TACACTGTTC GTGGGTGTCT CCTCCCCATC ACTGAGCTCC
1251 AGACACAGGG CTACCTCCTC G

Mousemagic (mouse ECSM4) amino acid sequence

EEVALCLESDGEETPTNSVSPMPRAP**SPPTYGYIS**IPTCGLADMGRAGGVGSEVGNLL
YPPRPCPTPT**SEGLANGWGSASEDNVPSARASLVSSSDGSFLAD****THFARALAVAVDSFGL**
SLDPREADCVF**TDASSPPSPRGDLSLTRSFSLPLWEWRPDWLEDAE**ISHTQRLGRGLPPWPP
DSRASSQRSWLTGAVPKAV

Figure 8 (page 1 of 2)

```

P1;T30805 - dutt1 protein - mouse
N;Alternate names: transmembrane receptor protein Robo1 homolog

SCORES  z-score: 292.0 E(): 3.3e-09
>>PIR2:T30805                                     (1612 aa)
Z-score: 292.0 expect(): 3.3e-09
Smith-Waterman score: 318;   28.0% identity in 472 aa overlap
(8-409:936-1383)

magic.pep      10      20      30
                MDHSDSQWLADTWRSTSGSRD-----LSSSSSLSS
T30805          PTVTYQRGGEAVSSGGRPGLLNISEPATQPLADTWPNNTGNNHNDCSINCCTAGNGNSDS
                910      920      930      940      950      960

magic.pep      40      50      60      70
                RLGADARDPLDCRRSLLSW-DSRSPGVPLLPDTSTFYGSL-----IAEL-----PSSTPA
T30805          NLTTYSR-PADCIANYNNQLDNKQTNL-MLPE-STVYGDVDLSNKNEMKTFNSPNLKDG
                970      980      990      1000      1010      1020

magic.pep      80      90      100      110      120      130
                R---PSPQ-VPVRRRLPPQLAQLSSPCSSDSLCSRRGLSSP---RLSLAPAE--AWKAK
T30805          RFVNPQGPQTPYATTQLIQ-ANLSNNMNGAGDSSEKHWKPPGQQKPEVAPIQYNNIMEQN
                1030      1040      1050      1060      1070      1080

magic.pep      140      150      160      170
                KKQELQHANS--PLLR-----GSHSLELRACEL-GNRGSKNLSQSPGAVPQALV
T30805          KLNKDYRANDTIPPTIPYNQSYDQNTGGSYNSSDRGSSSTSGSQGHKKGARTPKAPKQGGM
                1090      1100      1110      1120      1130      1140

```

Figure 8 (page 2 of 2)

magic.pep	180	190	200	210	220	230
T30805	AWRALGPKLLSSSNELVTRHLPAPLFFPHETPPTQSQQTQPPVAPQAPSSILLPAAPIPI					
	NWADL-----LPPPP--AHPPPHSNSEENMSVDESVDQEMPCPVPPAPM					
	1150	1160	1170	1180		
magic.pep	240	250	260	270	280	
T30805	LSPCSP-----PSPQASSLSGSPASSRLSSSSLSLGEDQDSVLTPEEVALCLE-					
	YLQDELQEEEDERGPTPPVRG-AASSPAAVSYSHQSTATLTPSPQEELQPM-LQDCPED					
	1190	1200	1210	1220	1230	1240
magic.pep	290	300	310	320	330	340
T30805	LSEGEETP---RNSVSPM--PRAPSPPTYGYISVPTASEF-TDMGRITGGVGPKGGVLL					
	LGHMPHPDRRRQPVSPPPPPRPISPPHTYGYISGPLVSDMDTDAPEEEEEDEADMEVAKM					
	1250	1260	1270	1280	1290	1300
magic.pep	350	360	370	380		
T30805	CPPRPCL-----TPTPS-----EGSLANGWGSASE-DNAASARASLVSSSDGSFLAD					
	QTRRLLLRGLLEQTPASSVGDLESSVTGSMINGWGSASEEDNISSGRSS-VSSSDGSFFTD					
	1310	1320	1330	1340	1350	1360
magic.pep	390	400	410			
T30805	AHFARALAVAVDSFGFGLFPPREADCVFIGM					
	ADFAQAVAAAAEYAGLKVARRQMQDAAGRHHFHASQCPRPTSPVSTDNSMSAVVIQKARP					
	1370	1380	1390	1400	1410	1420

Figure 9 (page 1 of 2)

T30805 - duttl protein - mouse
 N;Alternate names: transmembrane receptor protein Robol homolog
 C;Species: Mus musculus (house mouse)
 C;Date: 22-Oct-1999 #sequence_revision 22-Oct-1999 #text_change 22-Oct-1999
 C;Accession: T30805
 R;Wu, M.C.; Lowe, N.; Fordham, R.; Rabbitts, P. . . .

SCORES z-score: 299.4 E(): 1.3e-09
 >PIR2:T30805 (1612aa)
 Z-score: 299.4 expect(): 1.3e-09
 Smith-Waterman score: 263; 35.9% identity in 198 aa overlap
 (11-186:1251-1445)

		10	20	30	40
mousemagic.p		EEVALCLELSDGEETPTNSVSPMPRABPPPTYGYISPT			
		::			
T30805	TATLTSPQEELQPMQLQDCPEDLGHMPHPDPRRRQPV-SPPPPPPRPSPPHTYGYISGPL	1230	1240	1250	1260 1270
		50	60	70	80
mousemagic.p	CSSL-ADMGRAG-GVGSEVGN-----LLYPPRPCPTPTPSE-----GSLANGWGSAS				
	: : : : : : : : : : : : : :				
T30805	VSDMDTDAPEEEEDEADMEVAKMQTRRLRLRGLEQTTPASSVGDLESSVTSMINGWGSAS	1280	1290	1300	1310 1320 1330

	90	100	110	120	130	140
mousemagic.p	E-DNVPSARASL	VSSSDGSFLADTH	FARALAVAVDS	FGLSLDPRE---	ADCVFTDASSP	
T30805	EEDNISSGRSS-	VSSSDGSFFTAD	FAQAVAAAEEYAGL	KVARRQMDAAGR	RHHFHASQC	
	1340	1350	1360	1370	1380	1390

	150	160	170	180	190	
mousemagic.p	PSPRGDLSLTRFS-LPLWEWRPDWLEDAEISHTQRLGRG--LPPWPDPDRASSQRSWLT					
	: : : : : : : : :					
T30805	PRPTSPVSTDNMSAVVIQKARPAKKQHQPGLRREAYADDLPP-PPVPPPAAIKSPTVQ					
	1400	1410	1420	1430	1440	1450

200

mousemagic.p GAVPKAV

TT30805	SKAQLEVRPVMVPKIASIEARTDRSSDRKGSYKGREALDGRQVTDLRTNPSPDPREAQEQ				
1460	1470	1480	1490	1500	1510

Figure 10

Gap Weight: 8 Average Match: 2.912
 Length Weight: 2 Average Mismatch: -2.003

Quality: 597 Length: 135
 Ratio: 4.422 Gaps: 0
 Percent Similarity: 87.407 Percent Identity: 85.185

Match display thresholds for the alignment(s):

| = IDENTITY
 : = 2
 . = 1

mousemagic.pep x magic.pep September 12, 2000 18:05 ..

```

1 EEVALCLELSDGEETPTNSVSPMPRAPSPPTTYGYISIPTCSGLADMGRA 50
  |||||:|||||
280 EEVALCLELSEGEETPRNSVSPMPRAPSPPTTYGYISVPTASEFTDMGRT 329
  |||||.|||
51 GGGVGSEVGNLLYPPRPCPTPTPSEGLANGWGSASEDNVPSARASLVSS 100
  |||||.|||
330 GGGVGPKGGVLLCPPRPCLTPTPSEGLANGWGSASEDNAASARASLVSS 379
  |||||.|||
101 SDGSFLADTH FARALAVAVDSFGLSLDPREADCVF 135
  |||||:|||||
380 SDGSFLADAH FARALAVAVDSFGFGLPREADCVF 414
  |||||:|||||

```

Figure 11a

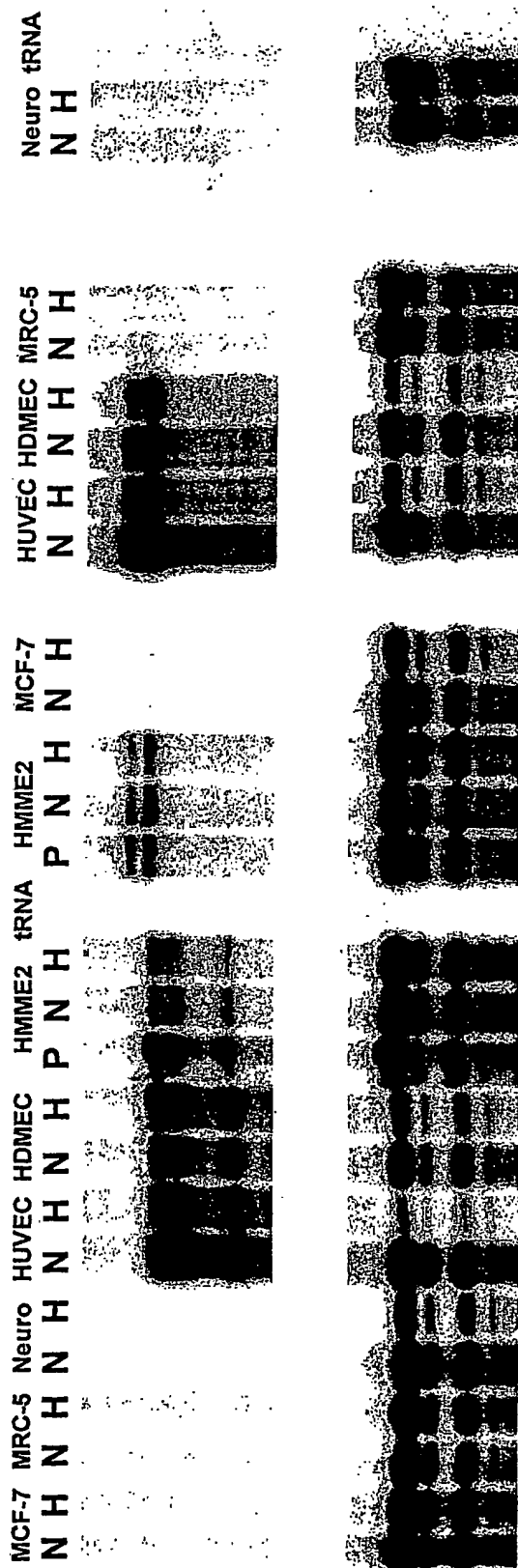


Figure 11b



Figure 12 (page 1 of 4)

```

1  GCGGCCGCGAATTCGGCACGAGCAGCAGGACAAAGTGCTCGGGACAAGGACATAGGGCTG  60
   -----+-----+-----+-----+-----+-----+
61  AGAGTAGCCATGGGCTCTGGAGGAGACAGCCTCCTGGGGGGCAGGGGTTCCCTGCCTCTG
   -----+-----+-----+-----+-----+-----+  120
      M G S G G D S L L G G R G S L P L
CTGCTCCTGCTCATCATGGGAGGCATGGCTCAGGACTCCCCGCCCCAGATCCTAGTCCAC
121  -----+-----+-----+-----+-----+-----+  180
      L L L L I M G G M A Q D S P P Q I L V H
CCCCAGGACCAGCTGTTCCAGGGCCCTGGCCCTGCCAGGATGAGCTGCCAAGCCTCAGGC
181  -----+-----+-----+-----+-----+-----+  240
      P Q D Q L F Q G P G P A R M S C Q A S G
CAGCCACCTCCCACCATCCGCTGGTTGCTGAATGGGCAGCCCCTGAGCATGGTGCCCCCA
241  -----+-----+-----+-----+-----+-----+  300
      Q P P P T I R W L L N G Q P L S M V P P
GACCCACACCACCTCCTGCCTGATGGGACCCTTCTGCTGCTACAGCCCCCTGCCGGGGA
301  -----+-----+-----+-----+-----+-----+  360
      D P H H L L P D G T L L L L Q P P A R G
CATGCCACGATGGCCAGGCCCTGTCCACAGACCTGGGTGTCTACACATGTGAGGCCAGC
361  -----+-----+-----+-----+-----+-----+  420
      H A H D G Q A L S T D L G V Y T C E A S
AACCGGCTTGGCACGGCAGTCAGCAGAGGCGCTCGGCTGTCTGTGGCTGTCTCCGGGAG
421  -----+-----+-----+-----+-----+-----+  480
      N R L G T A V S R G A R L S V A V L R E
GATTTCCAGATCCAGCCTCGGGACATGGTGGCTGTGGTGGGTGAGCAGTTTACTCTGGAA
481  -----+-----+-----+-----+-----+-----+  540
      D F Q I Q P R D M V A V V G E Q F T L E
TGTGGGCCGCCCTGGGGCCACCCAGAGCCACAGTCTCATGGTGGAAAGATGGGAAACCC
541  -----+-----+-----+-----+-----+-----+  600
      C G P P W G H P E P T V S W W K D G K P
CTGGCCCTCCAGCCCGGAAGGCACACAGTGTCCGGGGGGTCCCTGCTGATGGCAAGAGCA
601  -----+-----+-----+-----+-----+-----+  660
      L A L Q P G R H T V S G G S L L M A R A
GAGAAGAGTGACGAAGGGACCTACATGTGTGTGGCCACCAACAGCGCAGGACATAGGGAG
661  -----+-----+-----+-----+-----+-----+  720
      E K S D E G T Y M C V A T N S A G H R E
AGCCGCGCAGCCCGGGTTTCCATCCAGGAGCCCCAGGACTACACGAGCCTGTGGAGCTT
721  -----+-----+-----+-----+-----+-----+  780
      S R A A R V S I Q E P Q D Y T E P V E L
CTGGCTGTGCGAATTCAGCTGGAATAATGTGACACTGCTGAACCCGGATCCTGCAGAGGGC
781  -----+-----+-----+-----+-----+-----+  840
      L A V R I Q L E N V T L L N P D P A E G
CCCAAGCCTAGACCGGCGGTGTGGCTCAGCTGGAAGGTCAGTGGCCCTGCTGCGCCTGCC
841  -----+-----+-----+-----+-----+-----+  900
      P K P R P A V W L S W K V S G P A A P A
CAATCTTACACGGCCTTGTTTCAGGACCCAGACTGCCCCGGGAGGCCAGGGAGCTCCGTGG
901  -----+-----+-----+-----+-----+-----+  960
      Q S Y T A L F R T Q T A P G G Q G A P W
GCAGAGGAGCTGCTGGCCGGCTGGCAGAGCGCAGAGCTTGGAGGCCTCCACTGGGGCCAA
961  -----+-----+-----+-----+-----+-----+  1020
      A E E L L A G W Q S A E L G G L H W G Q
GACTACGAGTTCAAAGTGAGACCATCCTCTGGCCGGGCTCGAGGCCCTGACAGCAACGTG
1021 -----+-----+-----+-----+-----+-----+  1080
      D Y E F K V R P S S G R A R G P D S N V

```

Figure 12 (page 2 of 4)

CTGCTCCTGAGGCTGCCGGAAGTGGCCAGTGCCCCACCTCAGGAAGTGACTCTAAAG
1081 -----+-----+-----+-----+-----+ 1140
L L L R L P E K V P S A P P Q E V T L K
CCTGGCAATGGCACTGTCTTTGTGAGCTGGGTCCCACCACCTGCTGAAAACCAATGGC
1141 -----+-----+-----+-----+-----+ 1200
P G N G T V F V S W V P P P A E N H N G
ATCATCCGTGGCTACCAGGTCTGGAGCCTGGGCAACACATCACTGCCACCAGCCAACTGG
1201 -----+-----+-----+-----+-----+ 1260
I I R G Y Q V W S L G N T S L P P A N W
ACTGTAGTTGGTGAGCAGACCAGCTGGAAATCGCCACCCATATGCCAGGCTCCTACTGC
1261 -----+-----+-----+-----+-----+ 1320
T V V G E Q T Q L E I A T H M P G S Y C
GTGCAAGTGGCTGCAGTCACTGGTGCTGGAGCTGGGGAGCCAGTAGACCTGTCTGCCTC
1321 -----+-----+-----+-----+-----+ 1380
V Q V A A V T G A G A G E P S R P V C L
CTTTTAGAGCAGGCCATGGAGCGAGCCACCCAAGAAGCCAGTGAGCATGGTCCCTGGACC
1381 -----+-----+-----+-----+-----+ 1440
L L E Q A M E R A T Q E P S E H G P W T
CTGGAGCAGCTGAGGGCTACCTTGAAGCGGCCTGAGGTCATTGCCACCTGCGGTGTGCA
1441 -----+-----+-----+-----+-----+ 1500
L E Q L R A T L K R P E V I A T C G V A
CTCTGGCTGCTGCTTCTGGGCACCGCCGTGTGTATCCACCGCCGGCGCCGAGCTAGGGTG
1501 -----+-----+-----+-----+-----+ 1560
L W L L L L G T A V C I H R R R R A R V
CACCTGGGCCCAGGTCTGTACAGATATACCAGTGAGGATGCCATCCTAAAACACAGGATG
1561 -----+-----+-----+-----+-----+ 1620
H L G P G L Y R Y T S E D A I L K H R M
GATCACAGTGACTCCCAGTGTTGGCAGACACTTGGCGTTCCACCTCTGGCTCTCGGGAC
1621 -----+-----+-----+-----+-----+ 1680
D H S D S Q W L A D T W R S T S G S R D
CTGAGCAGCAGCAGCAGCCTCAGCAGTGGGCTGGGGGCGGATGCCCGGACCCACTAGAC
1681 -----+-----+-----+-----+-----+ 1740
L S S S S L S S R L G A D A R D P L D
TGTCGTCGCTCCTTGCTCTCCTGGGACTCCCGAAGCCCCGGCGTGCCCCCTGCTTCCAGAC
1741 -----+-----+-----+-----+-----+ 1800
C R R S L L S W D S R S P G V P L L P D
ACCAGCACTTTTATGGCTCCCTCATCGCTGAGCTGCCCTCCAGTACCCAGCCAGGCCA
1801 -----+-----+-----+-----+-----+ 1860
T S T F Y G S L I A E L P S S T P A R P
AGTCCCCAGGTCCCAGCTGTGAGGCGCCTCCCACCCAGCTGGCCAGCTCTCCAGCCCC
1861 -----+-----+-----+-----+-----+ 1920
S P Q V P A V R R L P P Q L A Q L S S P
TGTTCCAGCTCAGACAGCCTCTGCAGCCGAGGGGACTCTTCTCCCCGCTGTGCTCTG
1921 -----+-----+-----+-----+-----+ 1980
C S S S D S L C S R R G L S S P R L S L
GCCCCTGCAGAGGCTTGAAGGCCAAAAGAAGCAGGAGCTGCAGCATGCCAACAGTTCC
1981 -----+-----+-----+-----+-----+ 2040
A P A E A W K A K K K Q E L Q H A N S S
CCACTGCTCCGGGGCAGCCACTCCTTGGAGTCCGGGCCTGTGAGTTAGGAAATAGAGGT
2041 -----+-----+-----+-----+-----+ 2100
P L L R G S H S L E L R A C E L G N R G
TCCAAGAACCTTTCCCAAAGCCCAGGAGCTGTGCCCCAAGCTCTGGTTGCCTGGCGGGCC
2101 -----+-----+-----+-----+-----+ 2160
S K N L S Q S P G A V P Q A L V A W R A
CTGGGACCGAACTCCTCAGCTCCTCAAATGAGCTGGTTACTCGTCATCTCCCTCCAGCA
2161 -----+-----+-----+-----+-----+ 2220
L G P K L L S S S N E L V T R H L P P A

Figure 12 (page 3 of 4)

CCCTCTTTCTCATGAACTCCCCAACTCAGAGTCAACAGACCCAGCCTCCGGTGGCA
2221 -----+-----+-----+-----+-----+ 2280
P L F P H E T P P T Q S Q Q T Q P P V A
CCACAGGCTCCCTCCATCCTGCTGCCAGCAGCCCCATCCCCATCCTTAGCCCCCTGC
2281 -----+-----+-----+-----+-----+ 2340
P Q A P S S I L L P A A P I P I L S P C
AGTCCCCCTAGCCCCAGGCCTCTTCCCTCTCTGGCCCCAGCCAGCTTCCAGTCGCCTG
2341 -----+-----+-----+-----+-----+ 2400
S P P S P Q A S S L S G P S P A S S R L
TCCAGCTCCTCACTGTCATCCCTGGGGGAGGATCAAGACAGCGTGCTGACCCCTGAGGAG
2401 -----+-----+-----+-----+-----+ 2460
S S S S L S S L G E D Q D S V L T P E E
GTAGCCCTGTGCTTGGAACTCAGTGAGGGTGAGGAGACTCCAGGAACAGCGTCTCTCCC
2461 -----+-----+-----+-----+-----+ 2520
V A L C L E L S E G E E T P R N S V S P
ATGCCAAGGGCTCCTTCACCCCCACCACCTATGGGTACATCAGCGTCCCAACAGCCTCA
2521 -----+-----+-----+-----+-----+ 2580
M P R A P S P P T T Y G Y I S V P T A S
GAGTTCACGGACATGGGCAGGACTGGAGGAGGGGTGGGGCCCAAGGGGGAGTCTTGCTG
2581 -----+-----+-----+-----+-----+ 2640
E F T D M G R T G G G V G P K G G V L L
TGCCACCTCGGCCCTGCCTCACCCCCACCCCCAGCGAGGGCTCCTTAGCCAATGGTTGG
2641 -----+-----+-----+-----+-----+ 2700
C P P R P C L T P T P S E G S L A N G W
GGCTCAGCCTCTGAGGACAATGCCGCCAGCGCCAGAGCCAGCCTTGTCAGCTCCTCCGAT
2701 -----+-----+-----+-----+-----+ 2760
G S A S E D N A A S A R A S L V S S S D
GGCTCCTTCCTCGTGATGCTCACTTTGCCCGGCCCTGGCAGTGGCTGTGGATAGCTTT
2761 -----+-----+-----+-----+-----+ 2820
G S F L A D A H F A R A L A V A V D S F
GGTTTCGGTCTAGAGCCCAGGGAGGCAGACTGCGTCTTCATAGATGCCTCATCACCTCCC
2821 -----+-----+-----+-----+-----+ 2880
G F G L E P R E A D C V F I D A S S P P
TCCCCACGGGATGAGATCTTCTGACCCCCAACCTCTCCCTGCCCTGTGGGAGTGGAGG
2881 -----+-----+-----+-----+-----+ 2940
S P R D E I F L T P N L S L P L W E W R
CCAGACTGGTTGGAAGACATGGAGGTAGCCACACCCAGCGGCTGGGAAGGGGGATGCCT
2941 -----+-----+-----+-----+-----+ 3000
P D W L E D M E V S H T Q R L G R G M P
CCCTGGCCCCCTGAACTCTCAGATCTTCTCCAGAGAAGTCAGCTCCACTGTGCTATGCC
3001 -----+-----+-----+-----+-----+ 3060
P W P P E L S D L F P E K S A P L S Y A
CAAGGCTGGTGCTTCTCCTGTAGATTACTCTGAACCGTGTCCCTGAGACTTCCAGACG
3061 -----+-----+-----+-----+-----+ 3120
Q G W C F S C R L L L N R V P E T S Q T
GGAATCAGAACCACTTCTCCTGTTCCACCCACAAGACCTGGGCTGTGGTGTGGGTCTT
3121 -----+-----+-----+-----+-----+ 3180
G I R T T S P V P P T R P G L W C V G L
GGCCTGTGTTTCTCTGCAGCTGGGGTCCACCTTCCCAAGCCTCCAGAGAGTTCTCCCTCC
3181 -----+-----+-----+-----+-----+ 3240
G L C F S A A G V H L P K P P E S S P S
ACGATTGTGAAAACAAATGAAAACAAATTAGAGCAAAGCTGACCTGGAGCCCTCAGGGA
3241 -----+-----+-----+-----+-----+ 3300
T I V K T N E N K I R A K L T W S P Q G
GCAAAACATCATCTCCACCTGACTCCTAGCCACTGCTTTCTCCTCTGTGCCATCCACTCC
3301 -----+-----+-----+-----+-----+ 3360
A K H H L H L T P S H C F L L C A I H S

Figure 12 (page 4 of 4)

```
3361 CACCACCAGGTTGTTTTGGCCTGAGGAGCAGCCCTGCCTGCTGCTCTTCCCCCACCATT
-----+-----+-----+-----+-----+-----+ 3420
      H H Q V V L A *
3421 GGATCACAGGAAGTGGAGGAGCCAGAGGTGCCTTTGTGGAGGACAGCAGTGGCTGCTGGG
-----+-----+-----+-----+-----+ 3480

3481 AGAGGGCTGTGGAGGAAGGAGCTTCTCGGAGCCCCCTCTCAGCCTTACCTGGGCCCCCTCC
-----+-----+-----+-----+-----+ 3540

3541 TCTAGAGAAGAGCTCAACTCTCTCCCAACCTCACCATGGAAAGAAAATAATTATGAATGC
-----+-----+-----+-----+-----+ 3600

3601 CACTGAGGCACTGAGGCCCTACCTCATGCCAAACAAAGGGTTCAAGGCTGGGTCTAGCGA
-----+-----+-----+-----+-----+ 3660

3661 GGATGCTGAAGGAAGGGAGGTATGAGACCCGTAGGTCAAAAGCACCATCCTCGTA
-----+-----+-----+-----+-----+ 3715
```

Figure 13 (page 1 of 7)

(Linear) MAP of: /home/lif/icrt/mehtar/MuMR.seq check: 370 from: 1 to: 3688

REFORMAT of: MuMR.seq check: 370 from: 1 to: 3688 February 16, 2001 14:25

(No documentation)

February 16, 2001 15:01 ..

(Linear) MAP of: /home/lif/icrt/mehtar/MuMR.seq check: 370 from: 1 to: 3688

REFORMAT of: MuMR.seq check: 370 from: 1 to: 3688 February 16, 2001 14:25

(No documentation)

February 16, 2001 15:01 ..

```

      agtgtatgggacaaggagaggagccgagagcagccatgggctctggaggaacgggcctcc
1  -----+-----+-----+-----+-----+-----+ 60
      tcacataccctgttcctctcctcggtctcgtcggtacccgagacctccttgcccgagg

c      M G Q G E E P R A A M G S G G T G L L -

      tggggacggagtgccctctgcctctgctgctgcttttcatcatgggaggtgaggctctgg
61 -----+-----+-----+-----+-----+-----+ 120
      acccctgcctcaccggagacggagacgacgacgaaaagtagtaccctccactccgagacc

c      G T E W P L P L L L L F I M G G E A L D -

      attctccaccccagatcctagttcacccccaggaccagctacttcagggtcttgcccag
121 -----+-----+-----+-----+-----+-----+ 180
      taagaggtggggtctaggatcaagtgggggtcctgggtcgatgaagtcccagagaccgggtc

c      S P P Q I L V H P Q D Q L L Q G S G P A -

      ccaagatgaggtgcagatcatccggccaaccacctcccactatccgctgggtgctgaatg
181 -----+-----+-----+-----+-----+-----+ 240
      ggttctactccacgtctagtaggcccgttggtggagggtgataggcgaccgacgacttac

c      K M R C R S S G Q P P P T I R W L L N G -

      ggcagcccctcagcatggccaccccagacctacattaccttttgccggatgggaccctcc
241 -----+-----+-----+-----+-----+-----+ 300
      ccgtcggggagtcgtaccggtggggtctggatgtaatggaaaacggcctaccctggggagg

c      Q P L S M A T P D L H Y L L P D G T L L -

      tgttacatcgccctctgtccaggacggccacaagatgaccagaacatcctctcagcaa
301 -----+-----+-----+-----+-----+-----+ 360
      acaatgtagccgggagacaggtccctgcccgtgttctactgggtctttaggagagtcggt

c      L H R P S V Q G R P Q D D Q N I L S A I -

      tcctgggtgtctacacatgtgaggccagcaaccggctgggcacagcagtgagccgggggtg
361 -----+-----+-----+-----+-----+-----+ 420
      aggaccacagatgtgtacactccggtcggtggccgaccggtgctcactcggccccac
```

Figure 13 (page 2 of 7)

C L G V Y T C E A S N R L G T A V S R G A -
ctaggctgtctgtggctgtcctccaggaggacttccagatccaacctcgggacacagtgg
421 -----+-----+-----+-----+-----+ 480
gatccgacagacaccgacaggaggtcctcctgaagggtctagggttgagccctgtgtcacc

C R L S V A V L Q E D F Q I Q P R D T V A -
ccgtggtgggagagagacttggttcttgagtgtggctcctccctggggctacccaaaacct
481 -----+-----+-----+-----+-----+ 540
ggcaccaccctctctcgaaccaagaactcacaccaggagggaacccgatgggttttggga

C V V G E S L V L E C G P P W G Y P K P S -
cggctcatggtggaaagacgggaaaccttggtcctccagccaggaggcgacagtat
541 -----+-----+-----+-----+-----+ 600
gccagagtaccacctttctgccctttggggaccaggaggtcggtccctccgctgtcata

C V S W W K D G K P L V L Q P G R R T V S -
ctggggattccctgatggtgtcaagagcagagaagaatgactcggggacctatgtgtga
601 -----+-----+-----+-----+-----+ 660
gaccctaagggactaccacagttctcgtctcttcttactgagccctggatatacacat

C G D S L M V S R A E K N D S G T Y M C M -
tggccaccaacaatgctgggcaacgggagagccgagcagccagggtgtctatccaggaat
661 -----+-----+-----+-----+-----+ 720
accggtggttggttacgacccgttgccctctcggctcgtcggtcccacagataggtcctta

C A T N N A G Q R E S R A A R V S I Q E S -
cccaggaccacaaggaacatctagagcttctggctgttcgcattcagctggaaaatgtga
721 -----+-----+-----+-----+-----+ 780
gggtcctggtgttccttgtagatctcgaagaccgacaagcgtaagtcgaccttttact

C Q D H K E H L E L L A V R I Q L E N V T -
ccctgctaaacccgaaacctgtaaaaggtcccaagcctggggccatccgtgtggctcagct
781 -----+-----+-----+-----+-----+ 840
gggacgatttggggcttggtgacattttccagggttcggaccggtaggcacaccgagtcga

C L L N P E P V K G P K P G P S V W L S W -
ggaaggtagcgccctgctgcacctgctgagtcatacacagctctgttcaggactcaga
841 -----+-----+-----+-----+-----+ 900
ccttccactcgccgggacgacgtggacgactcagtatgtgtcgagacaagtcctgagtct

C K V S G P A A P A E S Y T A L F R T Q R -
ggtccccagggaaccaaggatctccatggacagaggtgctgctgctggcttgagagt
901 -----+-----+-----+-----+-----+ 960
ccaggggggtccctggttcctagaggtacctgtctccacgacgacgcaccgaacgtctcac

C S P R D Q G S P W T E V L L R G L Q S A -

Figure 13 (page 3 of 7)

```

          caaagcttgggggtctccactggggccaagactatgaattcaaagtgagaccgtcctccg
961  -----+-----+-----+-----+-----+-----+-----+ 1020
          gtttcgaacccccagaggtgaccccggttctgatacttaagtttctactctggcaggaggc

c      K  L  G  G  L  H  W  G  Q  D  Y  E  F  K  V  R  P  S  S  G  -

          gccgggctcgaggccctgacagcaatgtgttgctcctgaggctgcctgaacaggtgccca
1021 -----+-----+-----+-----+-----+-----+-----+ 1080
          cggccccgagctccgggactgtcgttacacaacgaggactccgacggacttgctccacgggt

c      R  A  R  G  P  D  S  N  V  L  L  L  R  L  P  E  Q  V  P  S  -

          gtgccccacctcaaggagtgaccttaagatctggcaacggtagtgtctttgtgagttggg
1081 -----+-----+-----+-----+-----+-----+-----+ 1140
          cacggggtggagttcctcactggaattctagaccgttgccatcacagaaacactcaacct

c      A  P  P  Q  G  V  T  L  R  S  G  N  G  S  V  F  V  S  W  A  -

          ctccaccacctgctgaaagccataatgggtgtcatccgtgggttaccaggctctggagcctgg
1141 -----+-----+-----+-----+-----+-----+-----+ 1200
          gaggtggtggacgactttcggtattaccacagtaggcaccaatgggtccagacctcggacc

c      P  P  P  A  E  S  H  N  G  V  I  R  G  Y  Q  V  W  S  L  G  -

          gcaatgcctcattgcctgctgccaactggaccgtagtgggtgaacagacccagctggaga
1201 -----+-----+-----+-----+-----+-----+-----+ 1260
          cgttacggagtaacggacgacggttgacctggcatcacccacttgtctgggtcgacctct

c      N  A  S  L  P  A  A  N  W  T  V  V  G  E  Q  T  Q  L  E  I  -

          tcgccacacgactgccaggctcctattgtgtgcaagtggctgcagtcactggagctgggtg
1261 -----+-----+-----+-----+-----+-----+-----+ 1320
          agcgggtgtgctgacggtccgaggataacacacggttcaccgacgtcagtgacctcgaccac

c      A  T  R  L  P  G  S  Y  C  V  Q  V  A  A  V  T  G  A  G  A  -

          ctgggagaactcagtacccctgtctgcctccttttagagcaggccatggagcaatcagcac
1321 -----+-----+-----+-----+-----+-----+-----+ 1380
          gacctcttgagtcatggggacagacggaggaaaaatctcgtccggtacctcgttagtcgtg

c      G  E  L  S  T  P  V  C  L  L  L  E  Q  A  M  E  Q  S  A  R  -

          gagaccccaggaaacatgttccctggaccctggaacagctgagggccaccttgagacgac
1381 -----+-----+-----+-----+-----+-----+-----+ 1440
          ctctggggtcctttgtacaagggtgggaccttgctcgactcccgggtggaactctgctg

c      D  P  R  K  H  V  P  W  T  L  E  Q  L  R  A  T  L  R  R  P  -

          cagaagtcattgccagtagtgctgtcctactctggttgctgctactaggcattactgtgt
1441 -----+-----+-----+-----+-----+-----+-----+ 1500
          gtcttcagtaacgggtcatcacgacaggatgagaccaacgacgatgatccgtaatgacaca

c      E  V  I  A  S  S  A  V  L  L  W  L  L  L  L  G  I  T  V  C  -

          gtatctacagacgacgcaaagctgggggtgcacctggggccaggtctgtacagatacacca
1501 -----+-----+-----+-----+-----+-----+-----+ 1560
          catagatgtctgctgcgtttcgacccacgtggacccgggtccagacatgtctatgtggt

```


Figure 13 (page 4 of 7)

C I Y R R R K A G V H L G P G L Y R Y T S -
gcgaggacgccattctaaaacacaggatggaccacagtgactcccatggctggcagaca
1561 -----+-----+-----+-----+-----+-----+ 1620
cgctcctgcggttaagattttgtgtcctacctggtgtcactgaggggtaccgaccgtctgt

C E D A I L K H R M D H S D S P W L A D T -
cctggcggttccacctctggctctcgagacctgagcagcagcagcagccttagtagtcggc
1621 -----+-----+-----+-----+-----+-----+ 1680
ggaccgcaaggtggagaccgagagctctggactcgtcgtcgtcgtcgggaatcatcagccg

C W R S T S G S R D L S S S S S L S S R L -
tgggattggaccctcgggacccactagagggcagggcgctccttgatctcctgggaccctc
1681 -----+-----+-----+-----+-----+-----+ 1740
accctaacctgggagccctgggtgatctcccgctccgaggaactagaggaccctgggag

C G L D P R D P L E G R R S L I S W D P R -
ggagccccggtgtacccctgcttccagacacgagcacggttttacggctccctcattgcag
1741 -----+-----+-----+-----+-----+-----+ 1800
cctcggggccacatggggacgaaggtctgtgctcgtgcaaatgccgagggagtaacgtc

C S P G V P L L P D T S T F Y G S L I A E -
agcagccttccagccctccagtcgggccaagcccaagacaccagctgctaggcgctttc
1801 -----+-----+-----+-----+-----+-----+ 1860
tcgtcgggaaggtcgggaggtcaggccggttcgggggttctgtggtcgacgatccgcgaaag

C Q P S S P P V R P S P K T P A A R R F P -
catccaagttggctggaacctccagccctgggctagctcagatagtctctgcagccgca
1861 -----+-----+-----+-----+-----+-----+ 1920
gtaggttcaaccgaccttgagggtcggggacccgatcgagtctatcagagacgtcggcgt

C S K L A G T S S P W A S S D S L C S R R -
ggggactctgttccccacgcatgtctctgaccctacagaggcttgggaaggccaaaaaga
1921 -----+-----+-----+-----+-----+-----+ 1980
cccctgagacaaggggtgcgtacagagactggggatgtctccgaaccttcgggtttttct

C G L C S P R M S L T P T E A W K A K K K -
agcaggaattgcaccaagctaacagctccccactgctccggggcagccaccccatggaaa
1981 -----+-----+-----+-----+-----+-----+ 2040
tcgtccttaacgtggttcgattgtcgaggggtgacgagggcccgctcgggtgggtacctt

C Q E L H Q A N S S P L L R G S H P M E I -
tctgggcctgggagttgggaagcagagcctccaagaacctttctcaaagcccaggagaag
2041 -----+-----+-----+-----+-----+-----+ 2100
agacccggaccctcaaccttcgtctcggaggttcttggaaagagtttcgggtcctcttc

C W A W E L G S R A S K N L S Q S P G E A -

Figure 13 (page 5 of 7)

cgccccgagccgtggtatcctggcggtgctgtgggaccacaacttcaccgcaactccagtg
2101 -----+-----+-----+-----+-----+-----+ 2160
gcggggctcggcaccataggaccgcacgacaccctgggtgttgaagtggcggttgagggtcac
C P R A V V S W R A V G P Q L H R N S S E -
agctggcatctcgtccactccctccaacacccctttctcttcgtggagcttccagtcagt
2161 -----+-----+-----+-----+-----+-----+ 2220
tcgaccgtagagcaggtgagggaggttgtgggaaagagaagcacctcgaaggtcagtac
C L A S R P L P P T P L S L R G A S S H D -
accacagagccagtgtgtggagaagctccaagctccctcctctgacccactgccagcag
2221 -----+-----+-----+-----+-----+-----+ 2280
tgggtgtctcgggtcacacacctcttcgaggttcgaggaggagactgggtgacgggtcgtc
C P Q S Q C V E K L Q A P S S D P L P A A -
cccctctctccgtcctcaactcttccagaccttccagcccccaggcctctttcctctcct
2281 -----+-----+-----+-----+-----+-----+ 2340
ggggagagagggcaggagttgagaaggtctggaaggtcgggggtcgggagaaaggagagga
C P L S V L N S S R P S S P Q A S F L S C -
gtcctagcccatcctccagcaacctgtccagctcctcgtgtcatccttagaggaggagg
2341 -----+-----+-----+-----+-----+-----+ 2400
caggatcgggtaggaggtcgttggacaggtcgaggagcgacagtaggaatctcctcctcc
C P S P S S S N L S S S S L S S L E E E E -
aggatcaggacagcgtgctcacccccgaggaggttagccctgtgtctggagctcagtgatg
2401 -----+-----+-----+-----+-----+-----+ 2460
tcctagtcctgtcgcacgagtggggggtcctccatcgggacacagacctcgagtcactac
C D Q D S V L T P E E V A L C L E L S D G -
gggaggagacacccacgaacagtgtatctcctatgccaaagagctccttccccgccaacaa
2461 -----+-----+-----+-----+-----+-----+ 2520
ccctcctctgtgggtgcttgtcacatagaggatacggttctcgaggaaggggcggttgtt
C E E T P T N S V S P M P R A P S P P T T -
cctatggctatatcagcataccaacctgtcaggactggcagacatgggcagagctggcg
2521 -----+-----+-----+-----+-----+-----+ 2580
ggataccgatatagtcgtatgggttgacgagtcctgaccgtctgtaccgctctcgaccgc
C Y G Y I S I P T C S G L A D M G R A G G -
ggggcggtgggggtctgaggttgggaacttactgtatccacctcgccctgccccaccctta
2581 -----+-----+-----+-----+-----+-----+ 2640
ccccgcaccccagactccaaccttgaatgacataggtggagccgggacgggggtggggat
C G V G S E V G N L L Y P P R P C P T P T -
caccacgagggggtccctggccaatggttgggggtcagcttctgaggacaatgtcccca
2641 -----+-----+-----+-----+-----+-----+ 2700
gtgggtcgtccccgagggacgggttaccaaccccgagtcgaagactcctgttacaggggt

Figure 13 (page 6 of 7)

c P S E G S L A N G W G S A S E D N V P S -
gcgccagggccagcctggtagctcttctgatggctccttcctcgctgatactcactttg
2701 -----+-----+-----+-----+-----+ 2760
cgcggtccccggtcggaccaatcgagaagactaccgaggaaggagcgactatgagtgaac

c A R A S L V S S S D G S F L A D T H F A -
ctcgtgccctggcagtggtgtggatagctttggcctcagtcctggatcccaggggaagctg
2761 -----+-----+-----+-----+-----+ 2820
gagcacgggaccgtcaccgacacctatcgaaaccggagtcagacctaggggtcccttcgac

c R A L A V A V D S F G L S L D P R E A D -
actgtgtcttctactgatgcctcatcacctccctccctcgggggtgatctctccctgaccc
2821 -----+-----+-----+-----+-----+ 2880
tgacacagaagtgactacggagtagtgaggaggaggaggagccactagagagggactggg

c C V F T D A S S P P S P R G D L S L T R -
gaagcttctctctgcctttgtgggagtgaggccagactggttggaagatgctgagatca
2881 -----+-----+-----+-----+-----+ 2940
cttcgaagagagacggaacacccctcacctccggtctgaccaaccttctacgactctagt

c S F S L P L W E W R P D W L E D A E I S -
gccacacccagaggctggggagggggctgcctccctggcctcctgattctagggcctctt
2941 -----+-----+-----+-----+-----+ 3000
cgggtgtgggtctccgacccctcccccgacggaggaggaccggaggactaagatcccggagaa

c H T Q R L G R G L P P W P P D S R A S S -
cccagcgaagttggctaactggtgctgtgcccaaggctggtgattcctcctgaattgtcc
3001 -----+-----+-----+-----+-----+ 3060
gggtcgcttcaaccgattgaccacgacacgggtccgaccactaaggaggacttaacagg

c Q R S W L T G A V P K A G D S S * -
ctgagaaggccagaagagcaccagaccactctcctgtctgtcccctggctttctcacat
3061 -----+-----+-----+-----+-----+ 3120
gactcttccggtcttctcgtgggtctggtgagaggacagacaggggaccgaaagagtgtgta

c
gtggagggtcttggcctatgcttctctgtaatagaagtccaccgtcactaggcttctggag
3121 -----+-----+-----+-----+-----+ 3180
cacctccagaaccggatacgaagagacattatcttcaggtggcagtgatccgaagacctc

c
agctctgtcattgggattgttaaaataaatgaaagcaaaccaaaatatgatcacgggagtg
3181 -----+-----+-----+-----+-----+ 3240
tcgagacagtaaccctaacaattttatttactttcgttttggttttatactagtgcctca

c

Figure 13 (page 7 of 7)

```

cttggattcccactgagaacaagacagcatcttcaggacagcagactctccacaaccaga
3241 -----+-----+-----+-----+-----+-----+ 3300
gaacctaaggggtgactcctgttctgtcgtagaaagtccgtcgtctgagaggtgttggtct

c -

acctttggcctaagtaagcctggctccggagctcccacctaagtggatcatggaaagaag
3301 -----+-----+-----+-----+-----+-----+ 3360
tggaaccggattcattcggaccgaggcctcgagggtggattcacctagtagctttcttc

c -

ggaagccaaccaggtcttcaggaaggacagaaatgttttttggtgagggctatggtggag
3361 -----+-----+-----+-----+-----+-----+ 3420
ccttcggttggtccagaagtccttcctgtcctttacaaaaaaccactcccgataccacctc

c M F F G E G Y G G G -

gacctgtggaagagccctctcatatctacttggactcctcccttagaggccagctcaacc
3421 -----+-----+-----+-----+-----+-----+ 3480
ctggacaccttctcgggagagtatagatgaacctgaggagggaatctccggtcgagttgg

c P V E E P S H I Y L D S S L R G Q L N P -

ctttcccagtcacaccatgcaaggaaactaaaggagaaaggtcgtggatgcagtgggcc
3481 -----+-----+-----+-----+-----+-----+ 3540
gaaaggggtcagtggttacgttcctttgatttcctctttccagcacctacgtcacccgg

c F P S H T M Q G N * -

ctatacagcgtcacagtcaatgcttcaaagtgagatcaatggaggagactgaaggaaagg
3541 -----+-----+-----+-----+-----+-----+ 3600
gatatgtcgcagtggtcagttacgaagtttcactctagttacctcctctgacttcctttcc

c M E E T E G K D -

acgcagggaacaggggaaccaatgcgctattctcattctaccgccactctgagcttaagg
3601 -----+-----+-----+-----+-----+-----+ 3660
tgcgtccctttgtcccttggttacgcgataagagtaagatggcgggtgagactcgaattcc

c A G K Q G T N A L F S F Y R H S E L K E -

aacttaattctataaaaactgtaaagacg
3661 -----+-----+-----+-----+-----+ 3688
ttgaattaagatattttgacatttctgc

c L N S I K L * -

```

Figure 14 (page 1 of 3)

BESTFIT OF: MR.PEP CHECK: 5275 FROM: 1 TO: 1104

TO: MUMR_1030818.PEP CHECK: 6771 FROM: 1 TO: 1228

TRANSLATE OF: MUMR.SEQ CHECK: 370 FROM: 3 TO: 3688

GENERATED SYMBOLS 1 TO: 1228.

REFORMAT OF: MUMR.SEQ CHECK: 370 FROM: 1 TO: 3688

SYMBOL COMPARISON TABLE:

/MOLBIO0/SOFTWARE/GCG/GCGCORE/DATA/RUNDATA/BLOSUM62.CMP

COMPCHK: 6430

GAP WEIGHT:	8	AVERAGE MATCH:	2.912
LENGTH WEIGHT:	2	AVERAGE MISMATCH:	-2.003

QUALITY:	4035	LENGTH:	1081
RATIO:	3.764	GAPS:	7
PERCENT SIMILARITY:	77.392	PERCENT IDENTITY:	74.390

MATCH DISPLAY THRESHOLDS FOR THE ALIGNMENT(S):

	=	IDENTITY
:	=	2
.	=	1

MR.PEP X MUMR.PEP

```

1  MSGGGDSLLGGRGSLPLLLLLLIMGGMAQDSPPQILVHPQDQLFQGP GPAR 50
   |||||  |||  |||||  ||||  |  |||||  |||||  |||  |||:
12 MSGGGTGLLGTEWPLPLLLLFIMGGEALDSPQILVHPQDQLLQSGPAK 61
   .
51 MSCQASGQPPPTIRWLLNGQPLSMVPPDPHLLPDGTLLLLLQPPARGHAH 100
   | |..|||||  |||||  |||  |  |:|||||  |||  .|  .|
62 MRCRSSGQPPPTIRWLLNGQPLSMATPDLHYLLPDGTLLLHRPSVQGRPQ 111
   .
101 DGQ.ALSTD LGVYTCEASNRLGTAVSRGARLSVAVLREDFQIQPRDMVAV 149
   | |  ||  |||||  |||||  |||||  |||||  |||||  |||||  |||
112 DDQNILSAILGVYTCEASNRLGTAVSRGARLSVAVLQEDFQIQPRDTVAV 161
   .
150 VGEQFTLECGPPWGHPEPTVSWWKDGKPLALQPGRHTVSGGSLLMARAEK 199
   |||  |||||  |||:|.|.|||||  ||||  ||||  |||:..|||
162 VGESLVLECGPPWGYPKPSVSWWKDGKPLVLQPGRRTVSGDSLMSRAEK 211
   .
200 SDEGTYMCVATNSAGHRESRAARVSIQEPQDYTEPVELLAVRIQLENVTL 249
   .|  |||||.|||.||  |||||  |||||  |||:  |  .|||||  |||||
212 NDSGTYMCMATNNAGQRESRAARVSIQESQDHKEHLELLAVRIQLENVTL 261

```

Figure 14 (page 2 of 3)

```

250 LNPDP AEGPKPRPAVWLSWKVSGPAAPAQSYTALFRTQTAPGGQGAPWAE 299
    |||:| .||| |.|||||:||||| |. ||.|| |
262 LNPEPVKGPKGPSVWLSWKVSGPAAPAESYTALFRTQ RSPRDQGS PWTE 311
300 ELLAGWQSAELGGLHWGQDYEFKVRPSSGRARGPDSNVLLRLPEKVPSA 349
    || | |||.|||||:||||| |.|||||
312 VLLRGLQSAKLGGLHWGQDYEFKVRPSSGRARGPDSNVLLRLPEQVPSA 361
350 PPQEVTLKPGNGTVFVSWVPPPAENHNGIIRGYQVWSLGN TSLPPANWTV 399
    ||| |||: |||.|||| | |||.|||:||||| ||| ||||
362 PPQGVTLRSGNGSVFVSWAPPPAESHNGVIRGYQVWSLGNASLPAANWTV 411
400 VGEQTQLEIATHMPGSYCVQVA AVTGAGAGEPSRPVCLLLEQAMERATQE 449
    |||||: ||||| | |||||.. :
412 VGEQTQLEIATRLPGSYCVQVA AVTGAGAGELSTPVCLLLEQAMEQSARD 461
450 PSEHG PWTLEQLRATLKRPEVIATCGVALWLLLLGTAVCIHRRRRARVHL 499
    | .| |||||:|||||. | ||||| |||:|||:| |||
462 PRKHVPWTLEQLRATLRRPEVIASSAVLLWLLLLGITVCIYRRRKAGVHL 511
500 GPGLYRYTSEDAILKHRMDHSDSQWLADTWRSTSGSRDLSSSSSLSSRLG 549
    |||||: ||||| | |||||
512 GPGLYRYTSEDAILKHRMDHSDSPWLADTWRSTSGSRDLSSSSSLSSRLG 561
550 ADARDPLDCRRSLLSWDSRSPGVPLLPDTSTFYGSLIAELPSSTPARPSP 599
    | ||||: ||||: || | ||||| ||| | ||||
562 LDPRDPLEGRRSLISWDPRSPGVPLLPDTSTFYGSLIAEQPSSPPVRPSP 611
600 QVPAVRRLPPQLAQLSSPCSSSDSLCSRRLSPRLSLAPAEAWKAKKKQ 649
    . || || | .|| || |.||||| |||: || | |||||
612 KTPAARRFPSKLAGTSSPWASSDSLCSRRLCSPRMSLTPTTEAWKAKKKQ 661
650 ELQHANS SPLLRGSHSLELRACELGNRGSKNLSQSPGAVPQALVAWRALG 699
    || | |||||: |: | |||. | ||||| |.|.|.|||. |
662 ELHQANS SPLLRGSHPMEIWAWELGSRASKNLSQSPGEAPRAVVSWRAVG 711
700 PKLLSSSNELVTRHLPPAPLFP HETPPTQSQQTQPPVAPQAPSSILLPAA 749
    |. | .|.|| |. ||| || |. | |||| ||||
712 PQLHRNSSELASRPLPPTPL.SLRGASSHD PQSQCVEKLQAPSSDPLPAA 760
750 PIPILSPCSPSPQASSLSGPSPASSRLSSSSSLSSL..GEDQDSVLTPEE 797
    |: |: |. | |||| || |||. || ||||| |||||
761 PLSVLNSSRPSSPQASFLSCPSPSSSNLSSSSSLSSLEEEEDQDSVLTPEE 810

```

Figure 14 (page 3 of 3)

```

798 VALCLELSEGEETPRNSVSPMPRAPSPPTTYGYISVPTASEFTDMGRTGG 847
   |||||:||||| ||||| ||||| ||||| |||||: || | ||| ||
811 VALCLELSDGEETPTNSVSPMPRAPSPPTTYGYISIPTCSGLADMGRAGG 860
   ||| . | || ||||| ||||| ||||| ||||| ||||| ||||| |||||
848 GVGPKGGVLLCPPRPCLTPTPSEGLANGWGSASEDNAASARASLVSSSD 897
   ||| . | || ||||| ||||| ||||| ||||| ||||| ||||| |||||
861 GVGSEVGNLLYPPRPCPTPTPSEGLANGWGSASEDNVPSARASLVSSSD 910
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
898 GSFLADAHFARALAVAVDSFGFGLPREADCVFIDASSPPSPRDEIFLTP 947
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
911 GSFLADTHFARALAVAVDSFGLSLDPREADCVFTDASSPPSPRGDLSLTR 960
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
948 NLSLPLWEWRPDWLEDMEVSHTQRLGRGMPPWPPELSDLFPEKSAPLSYA 997
   . ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
961 SFSLPLWEWRPDWLEDAEISHTQRLGRGLPPWPPD.....SRASSQRSWL 1005
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
998 QGWCFCRLLLRVPETSQTGIRTTSPVPPTTRPG.LWCVGLGLCFSAAGV 1046
   | ||| .. | |. || | ||||| |
1006 TGAVPKAGDSS*IVPEKAR...RAPRPLSCLSPGFLTTCGGLGLCFSVIEW 1052
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1047 ..HLPKPPESPSTIVKTNENKIRAKLTWSP 1075
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1053 HRH*ASGELCHWDC*NK*KQTKI*SRESWIP 1083

```

Figure 15 (page 1 of 8)

BESTFIT of: MR.seq check: 650 from: 1 to: 3715

REFORMAT of: MR.seq check: 650 from: 1 to: 3715 February 15, 2001 13:54

(No documentation)

to: MuMR.seq check: 370 from: 1 to: 3688

REFORMAT of: MuMR.seq check: 370 from: 1 to: 3688 February 16, 2001 14:25

(No documentation)

Symbol comparison table:

/molbio0/software/gcg/gcgcore/data/rundata/swgapdna.cmp

CompCheck: 2335

Gap Weight:	50	Average Match:	10.000
Length Weight:	3	Average Mismatch:	-9.000

Quality:	20259	Length:	3672
Ratio:	5.617	Gaps:	23
Percent Similarity:	79.169	Percent Identity:	79.169

Match display thresholds for the alignment(s):

| = IDENTITY

: = 5

. = 1

HuMR.seq x MuMR.seq February 16, 2001 14:38 ..

```

34 AGTGCTCGGGACAAGGACATAGGGCTGAGAGTAGCCATGGGCTCTGGAGG 83
   ||||  ||||||||| | | || ||||| |||||||||||||||||
  1 AGTGTATGGGACAAGGAGA.GGAGCCGAGAGCAGCCATGGGCTCTGGAGG 49

84 AGACAGCCTCCTGGGGGGCAGGGGTTCCTGCCTCTGCTGCTCCTGCTCA 133
   |   ||||||||| | | | ||||||||||||||| || |||
50 AACGGGCCTCCTGGGGACGGAGTGGCCTCTGCCTCTGCTGCTGCTTTTCA 99

134 TCATGGGAGGCATGGCTCAGGACTCCCCGCCCCAGATCCTAGTCCACCCC 183
   ||||||||| ||||| ||| || || ||||||||||||||| |||||
100 TCATGGGAGGTGAGGCTCTGGATTCTCCACCCCAGATCCTAGTTCACCCC 149

184 CAGGACCAGCTGTTCCAGGGCCCTGGCCCTGCCAGGATGAGCTGCCAAGC 233
   ||||||||| | ||||| ||||||| |||| ||||||| ||| | |
150 CAGGACCAGCTACTTCAGGGCTCTGGCCAGCCAAGATGAGGTGCAGATC 199

```


•

•

Q

•

2425 GGGGAGGATCAAGACAGCGTGCTGACCCCTGAGGAGGTAGCCCTGTGCTT 2474
| | | | | | | | | | | | | | | | | | | | | | |
2397 GAGGAGGATCAGGACAGCGTGCTCACCCCCGAGGAGGTAGCCCTGTGTCT 2446

2475 GGAACTCAGTGAGGGTGAGGAGACTCCCAGGAACAGCGTCTCTCCCATGC 2524
||| ||||| || ||||| ||||| ||||| || ||||| |||||
2447 GGAGCTCAGTGATGGGGAGGAGACACCACGAACAGTGTATCTCCTATGC 2496

2525 CAAGGGCTCCTTCACCCCCCACCACCTATGGGTACATCAGCGTCCCAACA 2574
|||| ||||| || || || ||||| || || ||||| | |||||
2497 CAAGAGCTCCTTCCCCGCCAACAACCTATGGCTATATCAGCATACCAACC 2546

2575 GCCTCAGAGTTCACGGACATGGGCAGGACTGGAGGAGGGGTGGGGCCCAA 2624
|||| | | ||||| ||||| ||||| || ||||| | |
2547 TGCTCAGGACTGGCAGACATGGGCAGAGCTGGCGGGGGCGTGGGGTCTGA 2596

2625 GGGGGGAGTCTTGCTGTGCCACCTCGGCCCTGCCTCACCCCCACCCCA 2674
|| || ||| |||| | ||||| ||||| ||||| || |||||
2597 GGTTGGGAACCTTACTGTATCCACCTCGGCCCTGCCCCACCCCTACACCCA 2646

2675 GCGAGGGCTCCTTAGCCAATGGTTGGGGCTCAGCCTCTGAGGACAATGCC 2724
||||||| || | ||||| ||||| ||||| ||||| ||||| ||
2647 GCGAGGGCTCCCTGGCCAATGGTTGGGGCTCAGCTTCTGAGGACAATGTC 2696

2725 GCCAGCGCCAGAGCCAGCCTTGTCAGCTCCTCCGATGGCTCCTTCCTCGC 2774
||||||| ||||| || ||||| || ||||| ||||| |||||
2697 CCCAGCGCCAGGGCCAGCCTGGTTAGCTCTTCTGATGGCTCCTTCCTCGC 2746

2775 TGATGCTCACTTTGCCCCGGGCCCTGGCAGTGGCTGTGGATAGCTTTGGTT 2824
|||| ||||| || || ||||| ||||| ||||| ||||| |||||
2747 TGATACTCACTTTGCTCGTGCCCTGGCAGTGGCTGTGGATAGCTTTGGCC 2796

2825 TCGGTCTAGAGCCCAGGGAGGCAGACTGCGTCTTCATAGATGCCTCATCA 2874
|| |||| || ||||| || ||||| ||||| ||||| |||||
2797 TCAGTCTGGATCCCAGGGAAGCTGACTGTGTCTTCACTGATGCCTCATCA 2846

2875 CCTCCCTCCCCACGGGATGAGATCTTCCTGACCCCCAACCTCTCCCTGCC 2924
||||||| |||| ||| || ||||| || || ||||| |||||
2847 CCTCCCTCCCTCGGGGTGATCTCTCCCTGACCCGAAGCTTCTCTCTGCC 2896

2925 CCTGTGGGAGTGAGAGGCCAGACTGGTTGGAAGACATGGAGGTGAGCCACA 2974
||||||| ||||| ||||| ||||| ||||| ||||| |||||
2897 TTTGTGGGAGTGAGAGGCCAGACTGGTTGGAAGATGCTGAGATGAGCCACA 2946

Figure 15 (page 8 of 8)

```

3515 CCTCTCAGCCTTACCTGGGCCCCTCCTCTAGAGAAGAGCTCAACTCTCT. 3563
      |||||      ||| ||| | ||||| ||||| ||||| ||| |
3436 CCTCTCATATCTACTTGGACTCCTCCCTTAGAGGCCAGCTCAACCCTTTC 3485
      .          .          .          .          .
3564 CCCAACCTCACCATGGAAAGAAAAT.AATTATGAATGCCACTGAGGCACT 3612
      |||| | ||||| || |||| | || | || | | || ||| |
3486 CCCAGTCACACCATGCAAGGAACTAAAGGAGAAAGGTCGTGGATGCAGT 3535
      .          .          .          .          .
3613 GAGGCCCTACCTCATGCCAAACAAAGGGTTCAAGGCTGGGTCTAGCGAGG 3662
      | | || | | | | || | || | ||||| | | || | ||||
3536 GGGCCCTATACAGCGTCACAGTCAATGCTTCAAAGTGAGATCAATGGAGG 3585
      .          .
3663 ATGCTGAAGGAAGGGAGGTATG 3684
      | ||||| |||| | | |
3586 AGACTGAAGGAAAGGACGCAGG 3607

```

Figure 16

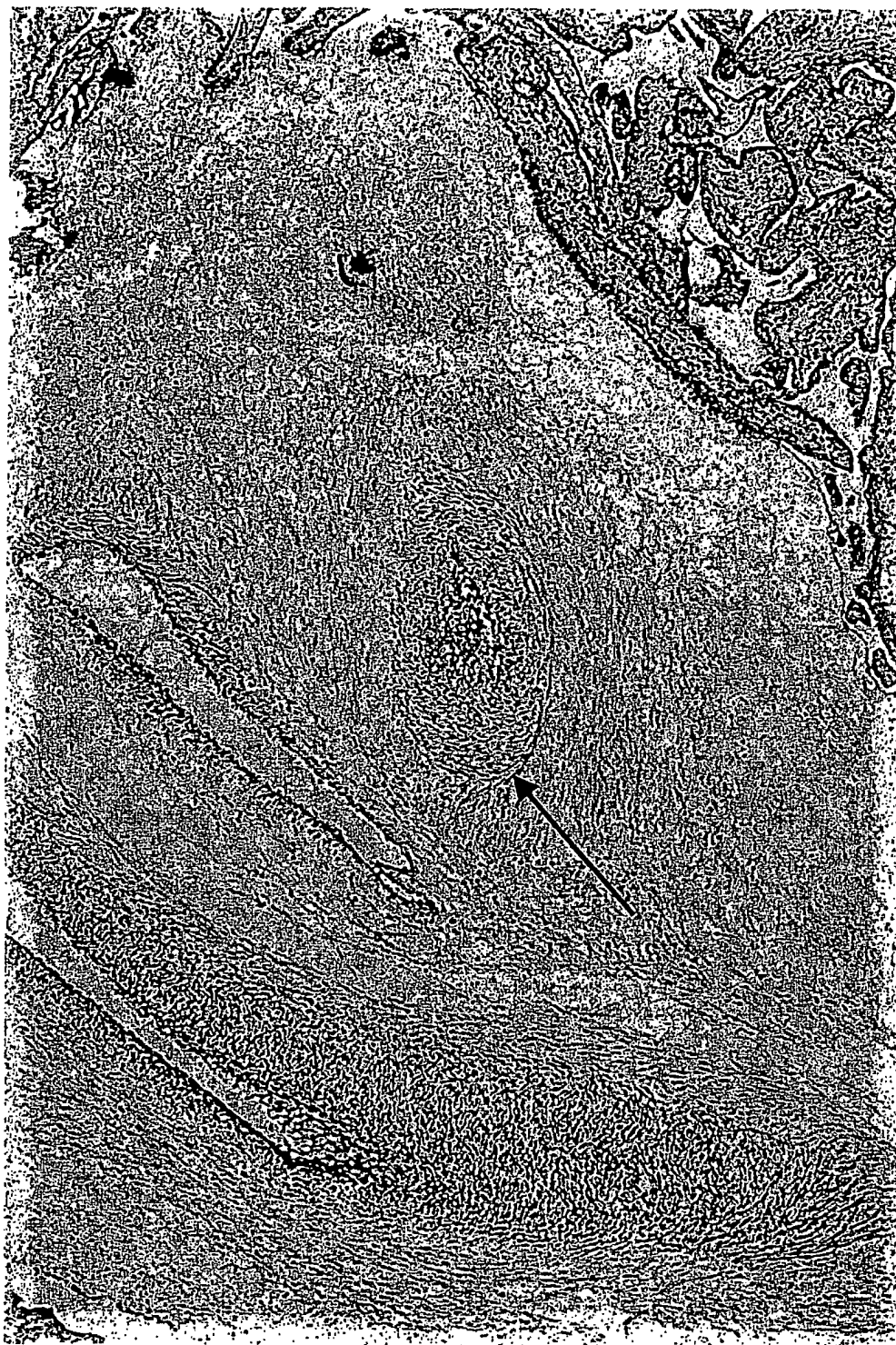


Figure 17

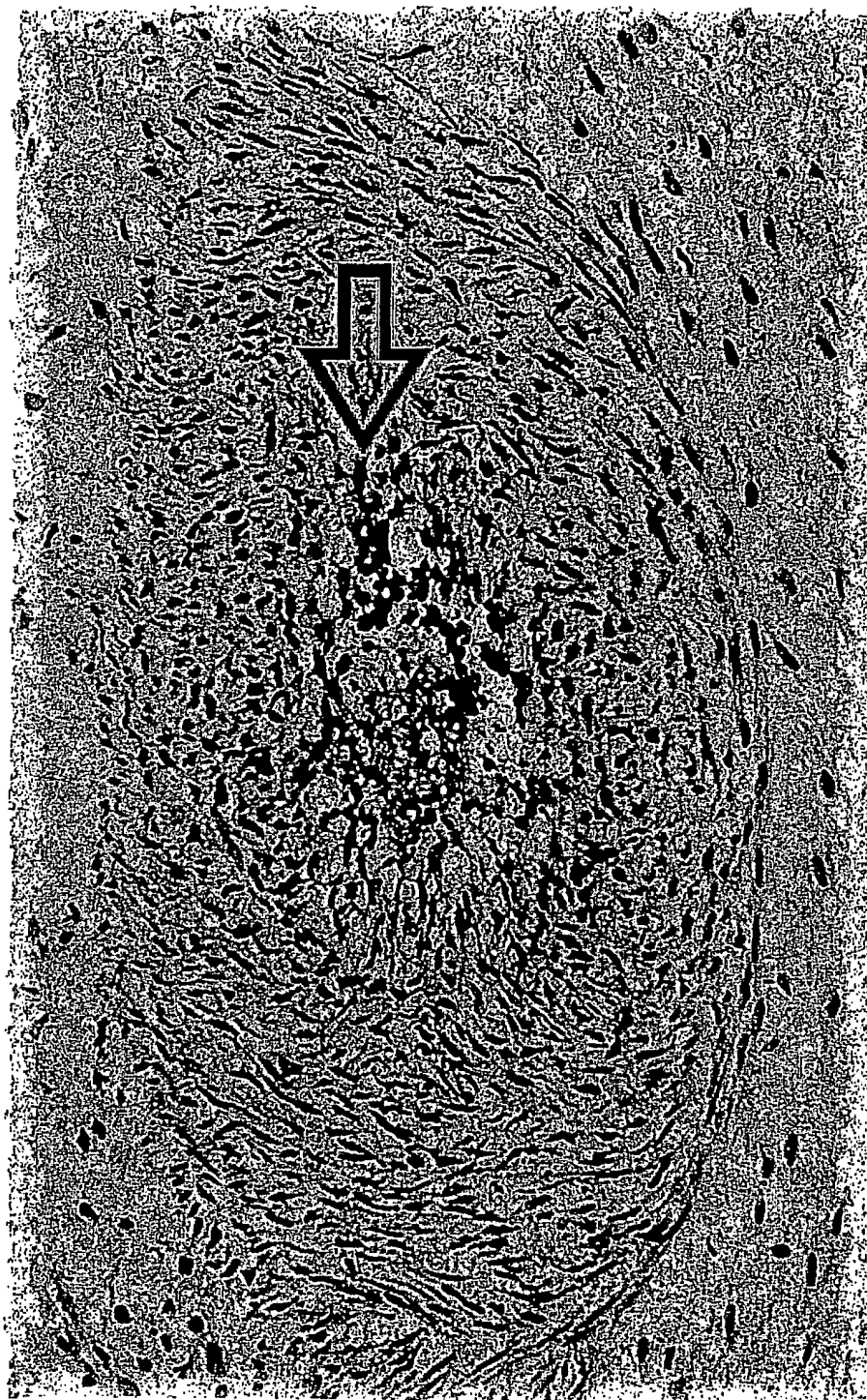


Figure 18

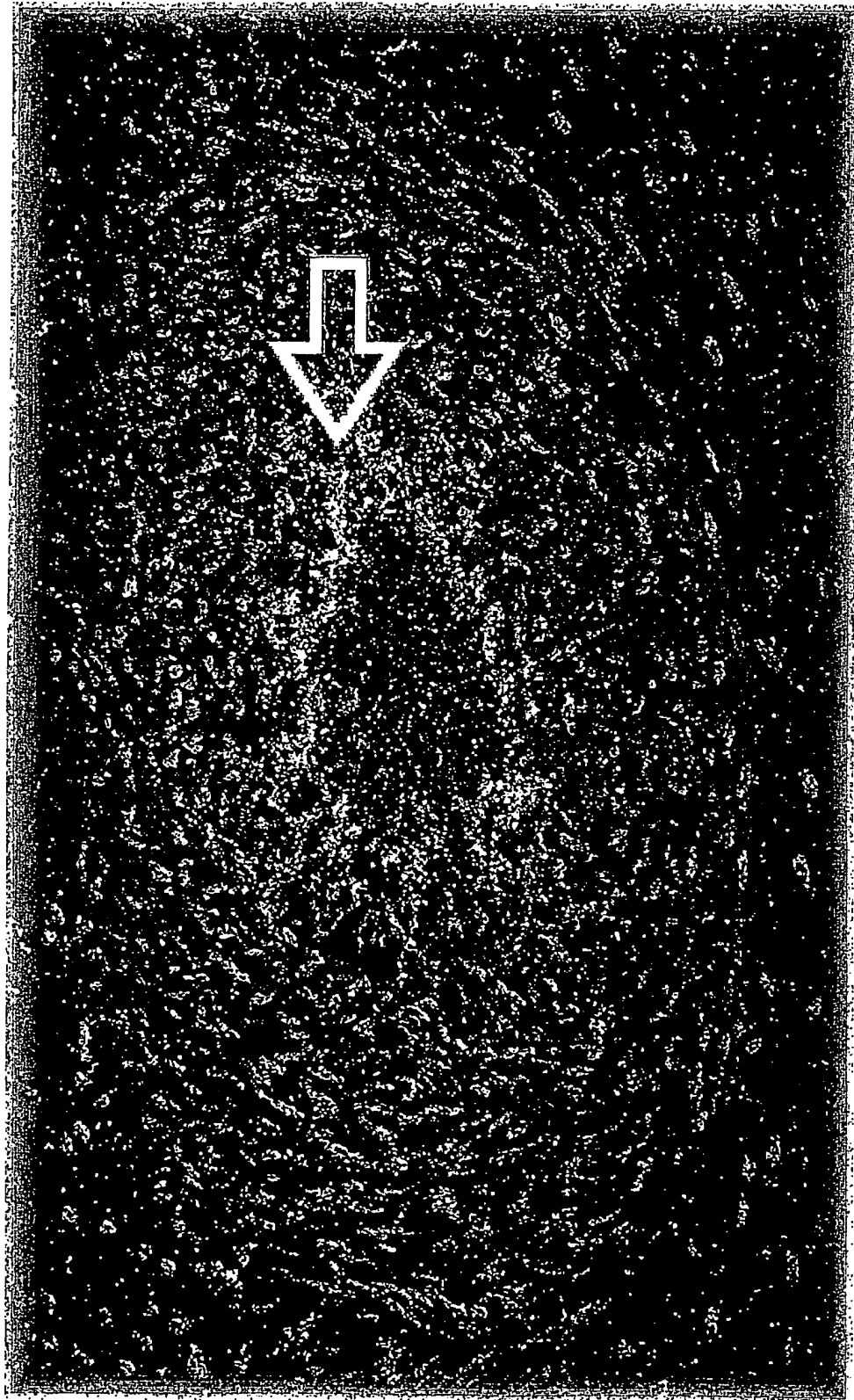


Figure 19

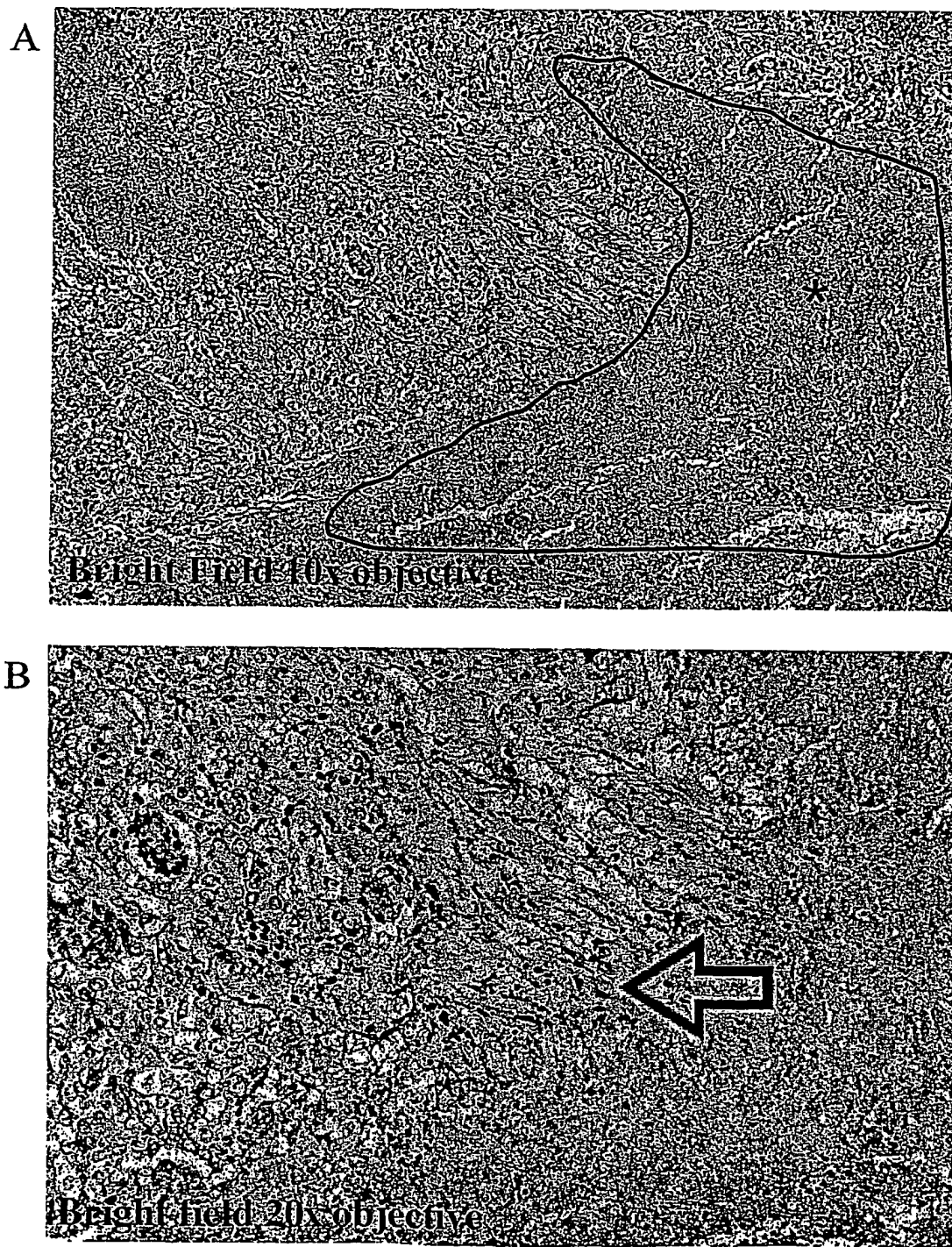
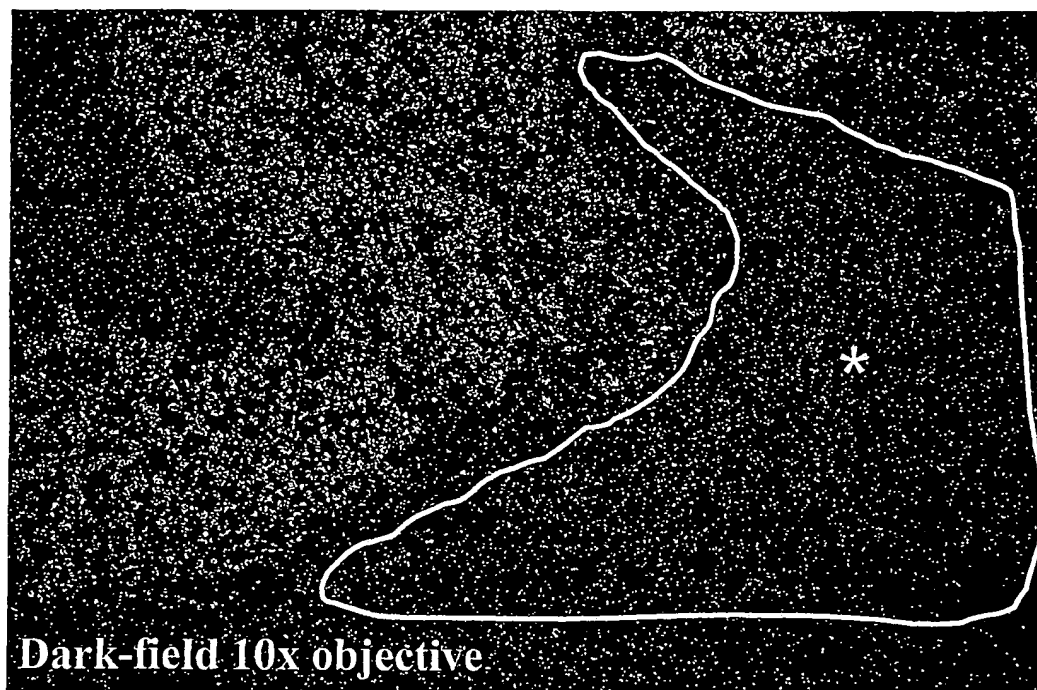
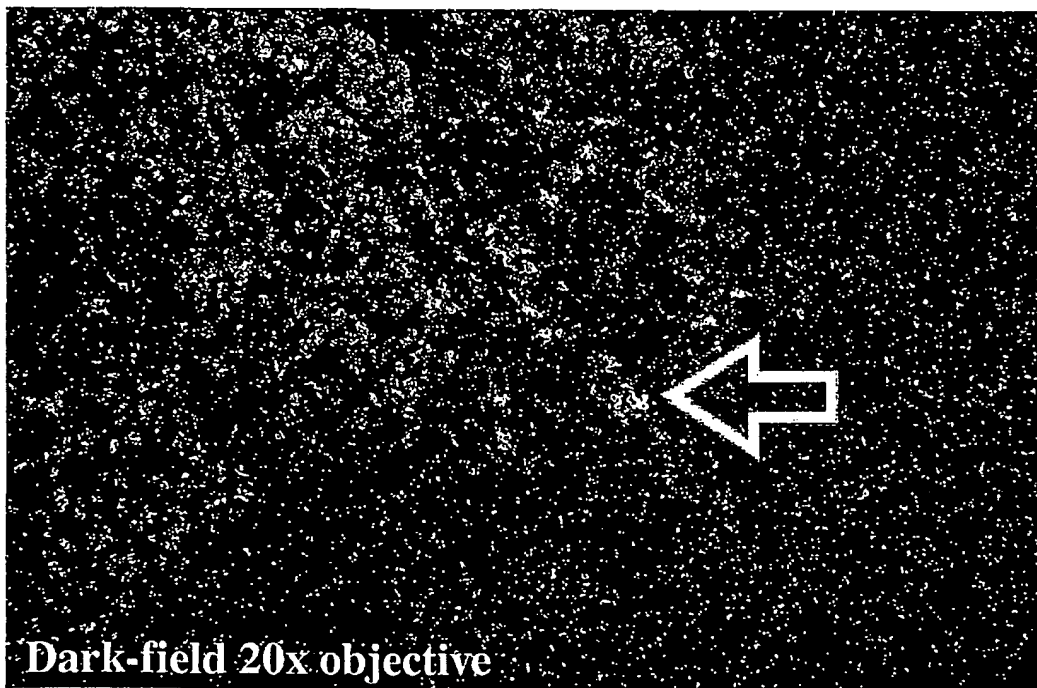


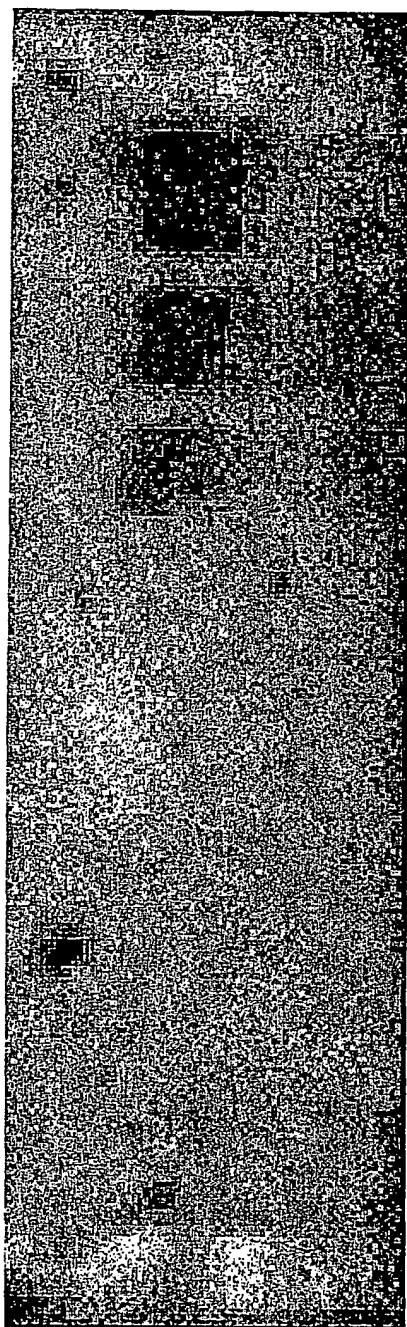
Figure 20

A



B





MR 165 (1:5000)

MR 165 (1:7500)

MR 165 (1:10000)

MR 311 (1:5000)

MR 311 (1:10000)

BSA 2mg/ml (1:1000)

MR 366 (1:2500)

MR 366 (1:5000)

Figure 21

Figure 22

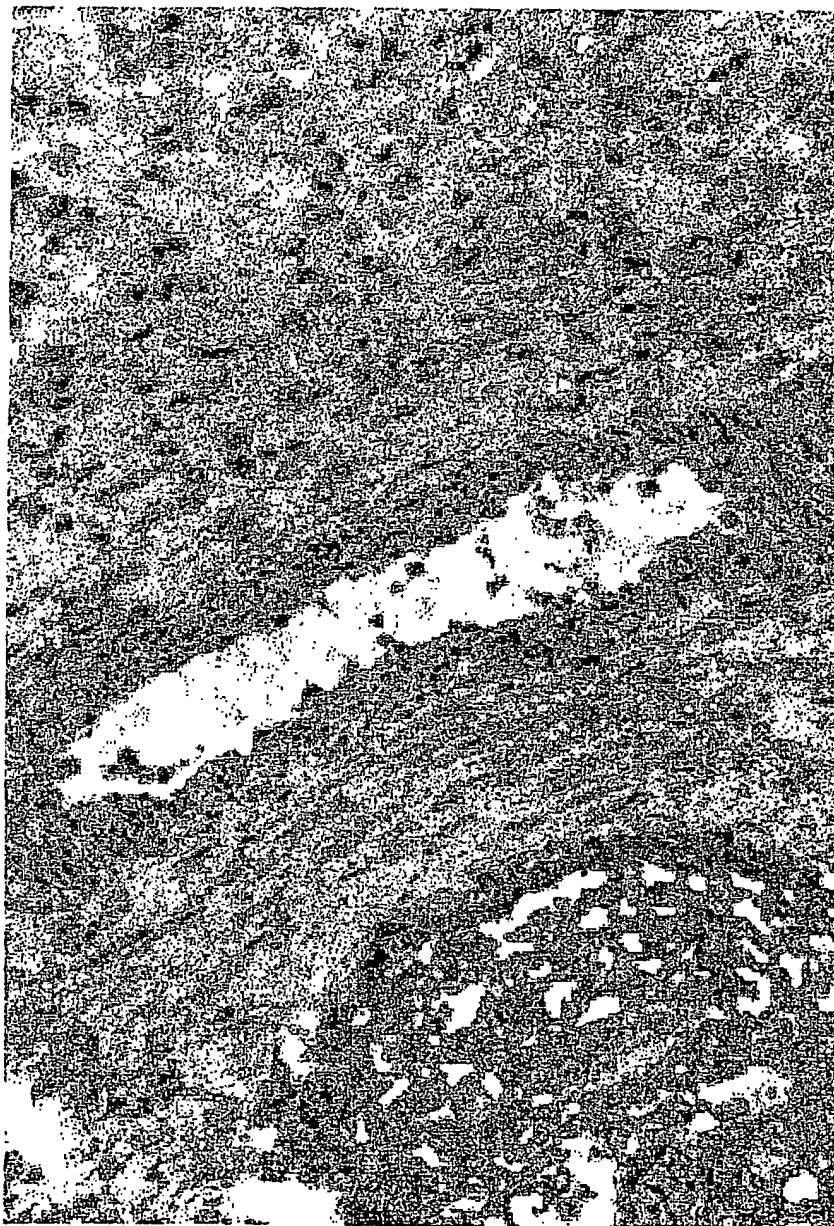


Figure 23

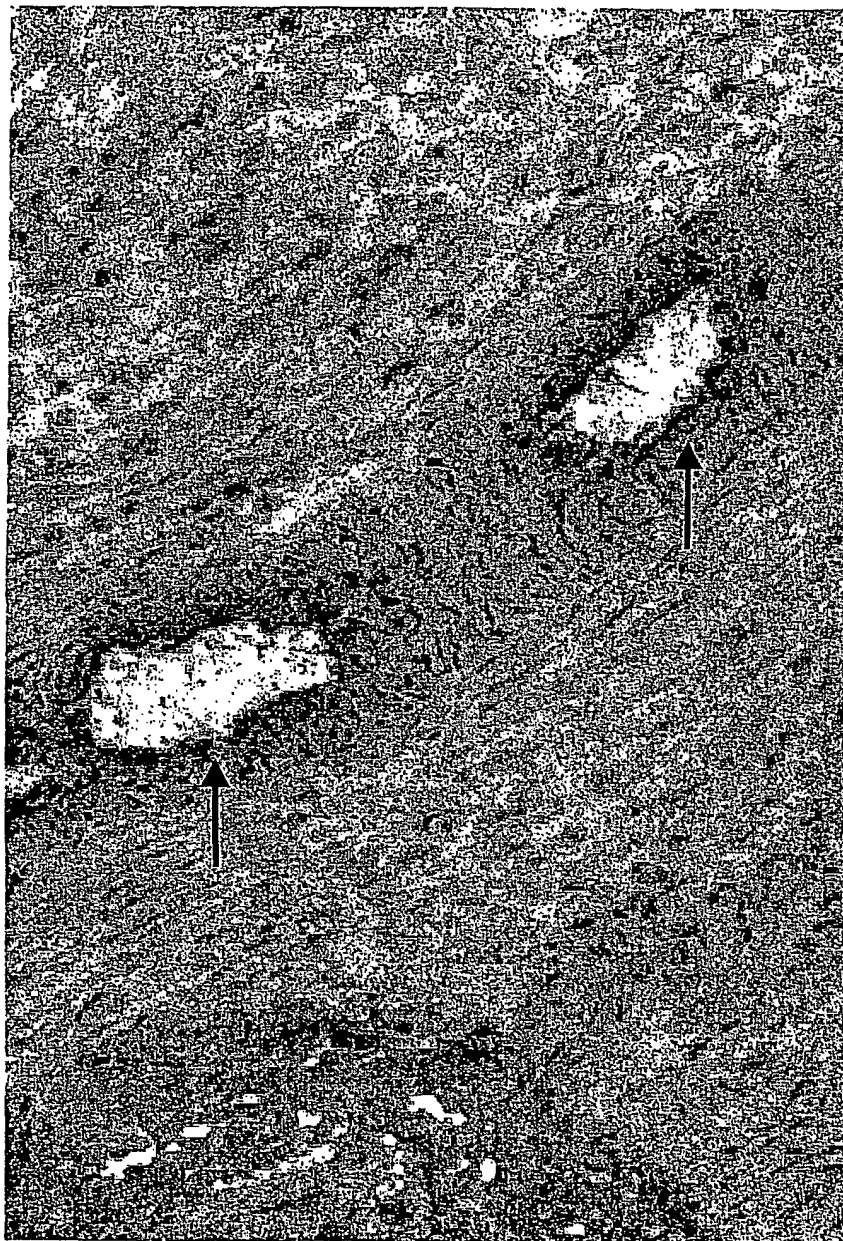


Figure 24

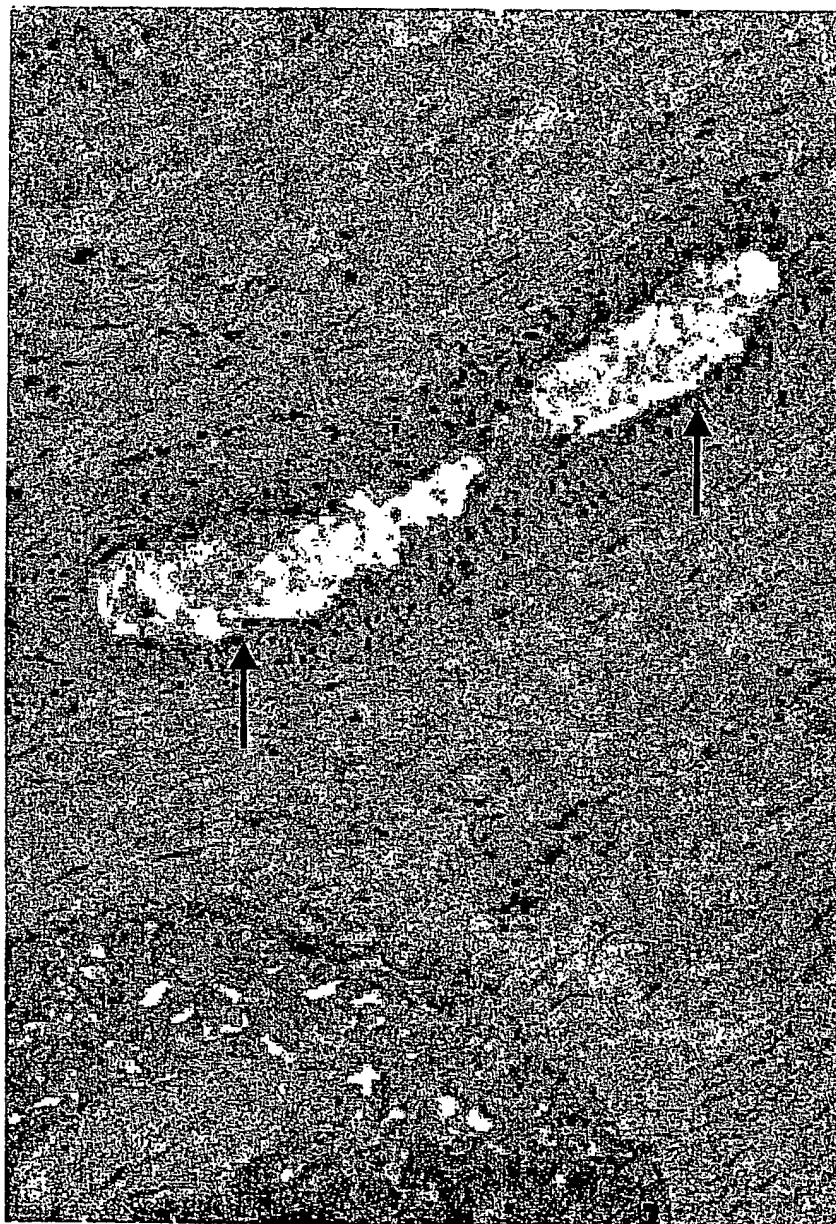


Figure 25

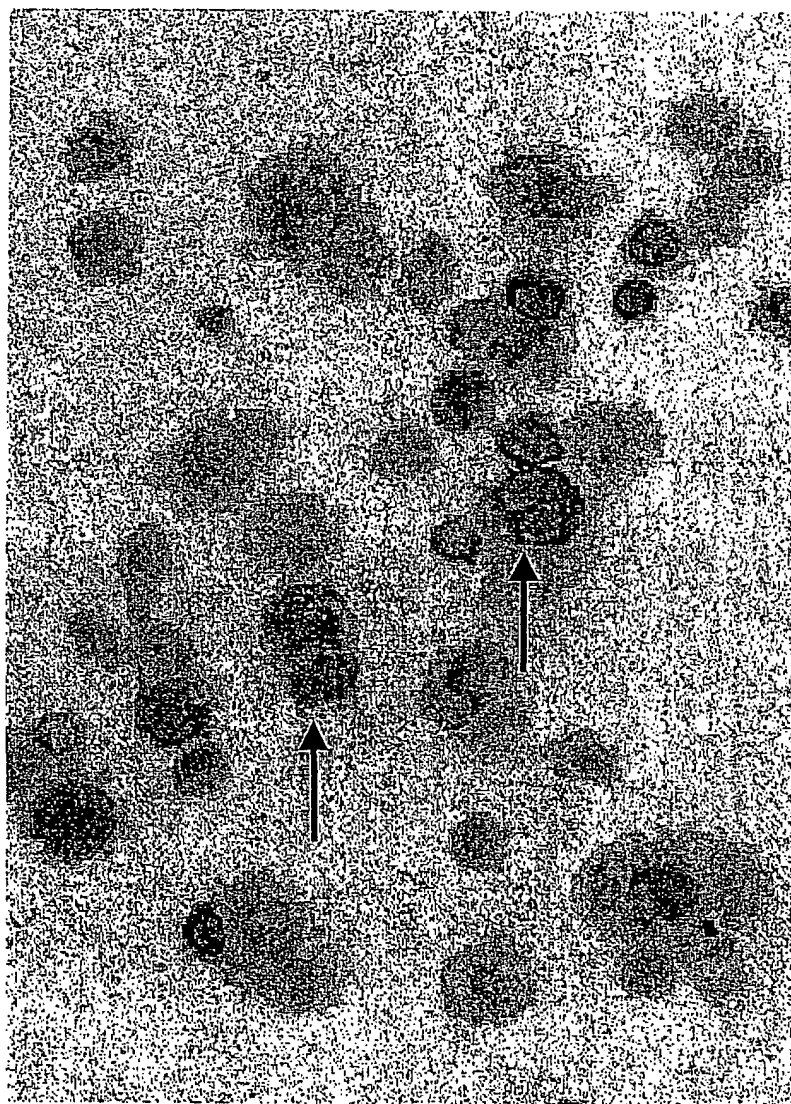


Figure 26

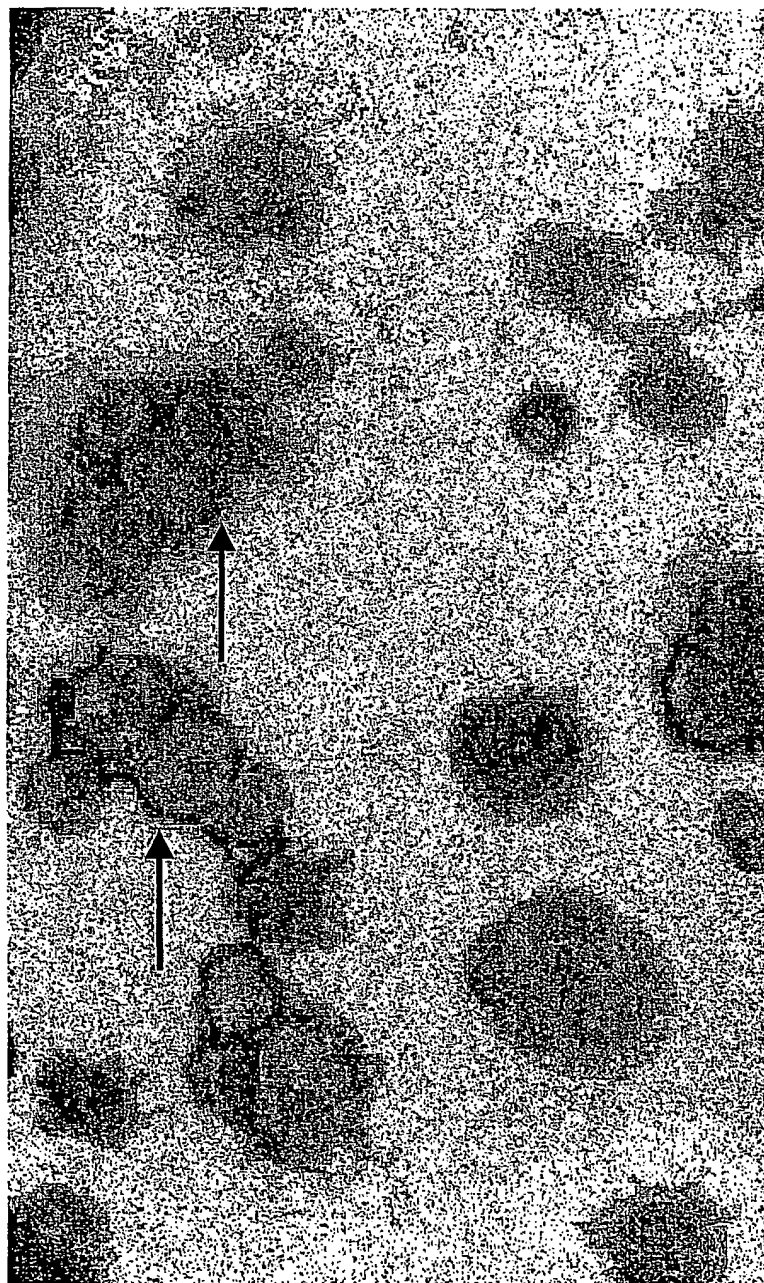


Figure 27 (page 1 of 2)

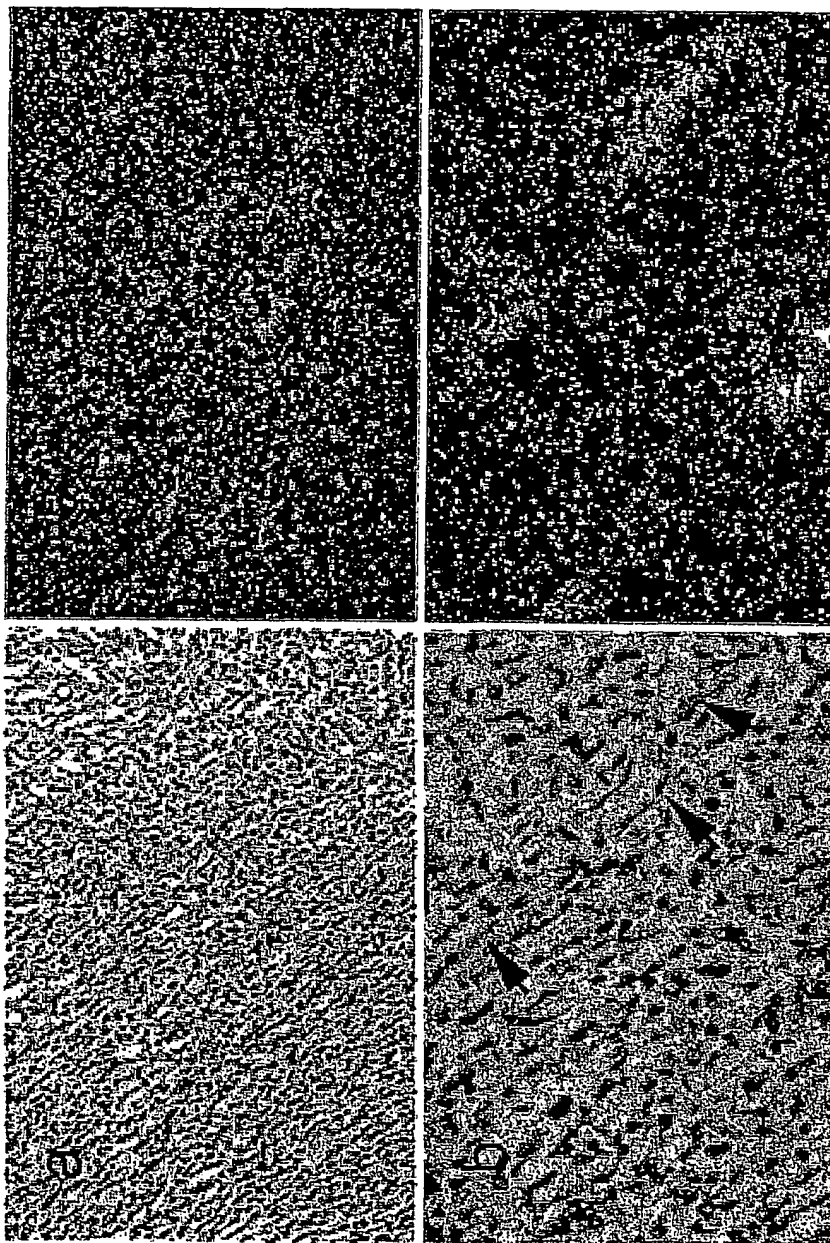
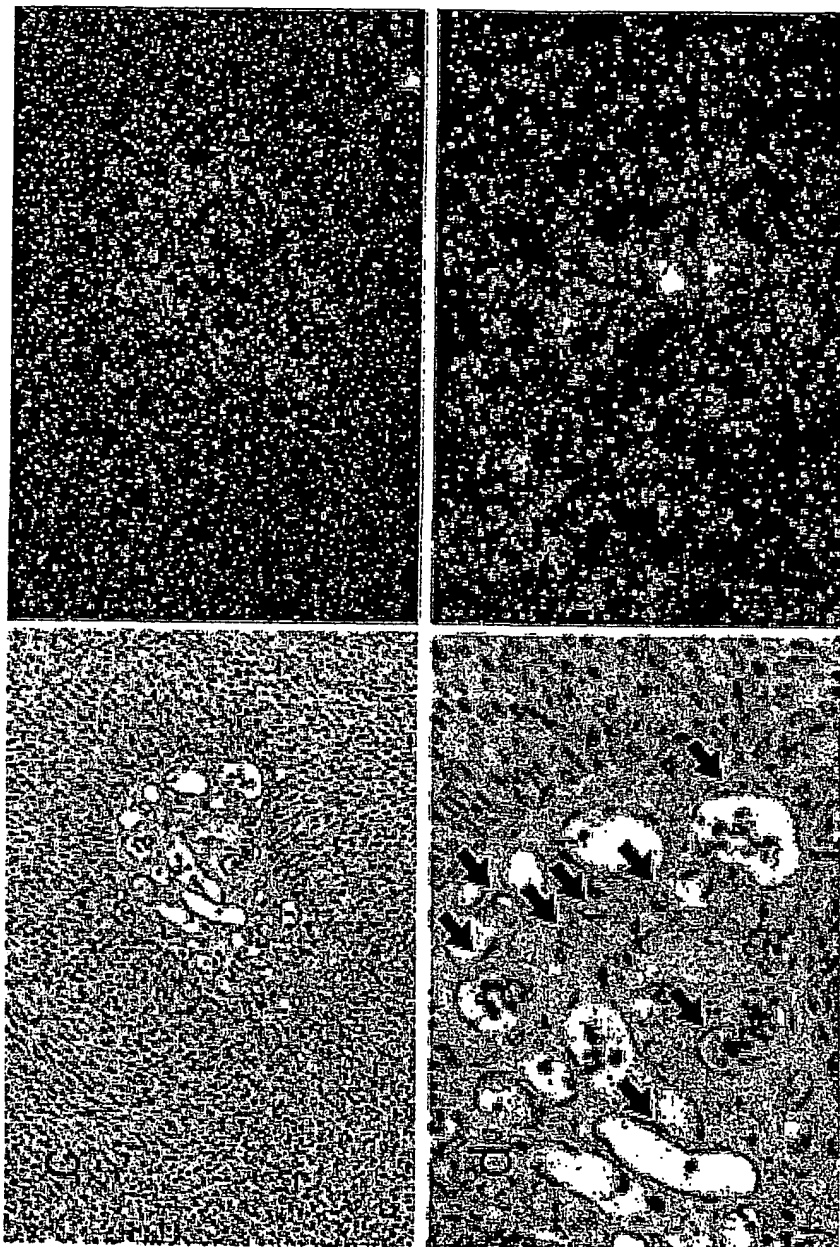


Figure 27 (page 2 of 2)



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